Department of Food and Nutrition
University of Helsinki
Finland

EKT-series 1836

Investigations on protein-lipid interactions under oxidative conditions

Göker Gürbüz

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public examination in Walter-hall, EE-building, Vikki on March 23rd, 2018, at 12 o'clock noon.

Helsinki, Finland 2018

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ISBN 978-951-51-4120-0 (paperback)
ISBN 978-951-51-4121-7 (PDF; http://ethesis.helsinki.fi)
ISSN 0355-1180

Unigrafia
Helsinki 2018

Gürbüz, G. 2018. Investigations on protein-lipid interactions under oxidative conditions. EKT-series 1836. University of Helsinki, Department of Food and Nutrition. 88 + 46 pp.

ABSTRACT

Oxidative reactions in food systems during processing and storage constitute a significant problem that determines the nutritional and sensory qualities of the food product as well as the textural and functional properties. Proteins and lipids, as essential components of foods, are highly prone to oxidative degradation that results in undesired modifications in food systems. Although lipid oxidation as a topic has been given a widespread attention, protein oxidation and its consequences in foods have been studied relatively recently. In particular, co-oxidation of food proteins and lipids, in terms of their interactions within the complex mechanism of oxidative reactions, has been gathering interest only lately. The behavior of proteins from various food sources and technological pre-treatments as well as the outcomes of this behavior under oxidative conditions in the presence of lipids is a much required subject on which to focus both by academia and industry.

This study investigated the oxidative modifications taking place in food proteins and lipids as well as their consequences in model systems. Firstly, molecular level changes between oxidized lipid product malondialdehyde (MDA) and β-lactoglobulin (β-Lg) peptides were characterized via utilization of LC-MS/MS techniques. The results showed that the main reactions occurred as formation of two Schiff base adducts between MDA and peptides located at either N-terminus amino groups or side-chains of amino acids. These adducts were identified as enaminal- and dihydropyridine-type derivatives that were observed with +54 and +134 Da mass increments of native peptides, respectively.

Following studies focused on emulsion model systems that were stabilized by plant proteins in varying compositions. Emulsions were stored at different temperatures during which oxidation of lipids and proteins was monitored. Emulsions prepared with quinoa and amaranth proteins were compared to Tween®20-stabilized emulsions. Quinoa protein-emulsions showed the least oxidative and physical stability due to rapid protein degradation while oxidation of amaranth proteins proceeded in a slower fashion. Tween®20-stabilized emulsions exhibited higher oxidative and physical stability compared to protein-stabilized emulsions.

Final model study focused on the oxidation of continuous and interfacial proteins in emulsions prepared with faba bean proteins that had undergone microwave (MWT) and conventional thermal treatment (CTT) in order to inhibit native lipoxygenase enzyme activity. Enzymatic oxidation pathway had a profound effect on the extent of oxidation. Emulsions prepared with CTT proteins exhibited higher stability than MWT protein-stabilized emulsions. Furthermore, degradation of interfacial proteins was more emphasized than those located in the aqueous phase in both MWT and CTT emulsions. In contrast, emulsions prepared with proteins from untreated faba beans contained highly oxidized aqueous phase proteins due to extensive enzymatic oxidation.

This thesis demonstrates that interdependent relations between proteins and lipids such as adduct formation, free-radical transfer, and reactions between oxidized species have a significant effect on the overall course of oxidation of the food system which affect the observed modifications. Therefore, customized solutions against oxidation should regard the intricate relations of protein-lipid co-oxidation in a food system that contains proteins and lipids as major constituents.

PREFACE

The research reported in this dissertation was carried out at the Department of Food and Nutrition (formerly Department of Food and Environmental Sciences) of the University of Helsinki. The work was funded by the ABS Graduate School, the Finnish Cultural Foundation and the Agricultural Research Foundation of August Johannes and Aino Tiura. I gratefully acknowledge their financial support.

I am sincerely thankful for my supervisor Prof. Marina Heinonen without whose continuous support and encouragement I would not be able to become an author of a doctoral dissertation and accomplish whatever I did in academia. Her door was always open to me no matter how busy she was and always lent an ear to me at times that I was doubting myself. I would also like to thank Prof. Vieno Piironen for her guidance and leadership along the years.

I am grateful to the pre-examiners of this dissertation, Assoc. Prof. Mario Estévez and Doc. Riitta Partanen, who provided me with valuable comments and suggestions to shape the thesis in the best way possible.

I would like to use this opportunity to thank the collaborators and co-authors whose knowledge and skills made it possible for this work to be completed: Doc. Kirsi Jouppila, Doc. Tuula Sontag-Strohm, Dr. Jose Martin Ramos Diaz, Marjo Pulkkinen, Zhong-qing Zhao, Chang Liu and Vilja Kauntola. My gratitude extends further to the research and teaching staff of Food Chemistry Division who were always happy to share their expertise and who, starting from my Master's degree years, made a food chemist out of me: Doc. Anna-Maija Lampi, Doc. Velimatti Ollilainen and Doc. Susanna Kariluoto. I would also like to take the time to give thanks to one of my oldest colleagues in this division and former Master's thesis supervisor Dr. Tuuli Koivumäki who was always ready to listen to me babbling about simply anything and helping me out whenever I was in need of information or support. I am also indebted to other former and current research staff: Assistant Prof. Kirsi Mikkonen, Dr. Mari Lehtonen, Dr. Minnamari Edelmann, Dr. Annelie Damerau, Dr. Bhawani Chamlagain, Dr. Petri Kylli, Bei Wang and Zhen Yang. Much of the work reported in this book would be very difficult to conceive without the help of our skillful technical staff who were always accommodating and patient with me: Miikka Olin, Taru Rautavesi, Maija Ylinen and Timo Holopainen. Other "Viikki" people that I have shared my everyday life and with whom I had countless conversations both over a cup of coffee and a glass of beer, also deserve my deepest thanks: Dr. Paulina Deptuła, Dr. Jose Martin Ramos Diaz, Dr. Kevin Deegan and Pasi Perkiö. I have very much enjoyed the time spent, work carried out and many

doses of caffeine consumed in the company of these above-mentioned "työkaverit".

Every person that has aimed to achieve a doctoral degree is aware that this kind of engagement becomes an inseparable part of their life. So, just like PhD work affected my "other" life, my "other" life also affected how I dealt with the feelings of excitement, stress and captivation related to my doctoral candidacy. This is where I would like to extend a big, heartfelt "Thank you" to my friends outside of academia who will remain nameless here just because of the simple reason that there are way too many to list. I shared and experienced with them intimate conversations, stimulating arguments, delights of Finnish summer, amusing trips, many midsummer bonfires, and the joy of connection through dancing Lindy hop to jazz tunes. I cherish all the memories.

As cliché as it may sound but as true as it can get, I couldn't be here without my parents, Gül and Mehmet Gürbüz. When I had embarked upon this path that left behind the country where I grew up, they gave me their full support although it saddened them to part from their son. I appreciate that they are always there for me. I also want to thank my brother Güney who is ready to listen to me anytime and eager to talk about all the movies we both enjoy.

Finally, I would like to express my gratification for being able to witness this existence of nature and life, the phenomena of which make a perpetual student out of me that stands in admiration of their awe. Our experiences of life and nature, as they come, are the essence of this journey that shapes us into who we are, how we think and what meaning we assign to reality. So, I'm glad to allow myself feel what I feel through these experiences and be who they turn me into, just because that is what I believe to be the purpose of it all: To live and to learn.

Helsinki, March 2018

Göker Gürbüz

"It is good to have an end to journey toward; but it is the journey that matters, in the end."

— Ursula K. Le Guin

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LIST OF ORIGINAL PUBLICATIONS

I Gürbüz G, Heinonen M. 2015. LC–MS investigations on interactions between isolated β-lactoglobulin peptides and lipid oxidation product malondialdehyde. Food Chemistry 175:300-305.

II Gürbüz G, Kauntola V, Ramos Diaz JM, Jouppila K, Heinonen M. 2018. Oxidative and physical stability of oil–in–water emulsions prepared with quinoa and amaranth proteins. European Food Research and Technology 244:469-479.

III Gürbüz G, Liu C, Jiang Z-Q, Pulkkinen M, Piironen V, Sontag-Strohm T, Heinonen M. Protein-lipid co-oxidation in emulsions stabilized by microwave- and conventional thermal-treated faba bean proteins. *Submitted*.

 The publications are referred to in the text by their roman numerals.

Contribution of the author to papers I to III:

I Göker Gürbüz planned the study together with M. Heinonen. He was responsible for the experimental work. He had the main responsibility for interpreting the results, preparing the manuscript and was the corresponding author of the paper.

II Göker Gürbüz planned the study together with the other authors. The experimental work was conducted partly as a Master's thesis work of V. Kauntola. Göker Gürbüz had the main responsibility in supervision of the experimental work, interpreting the results and preparing the manuscript. He was the corresponding author of the paper.

III Göker Gürbüz planned the study together with the other authors. The experimental work was conducted partly as a Master's thesis work of C. Liu. Göker Gürbüz had the main responsibility in supervision of the experimental work, interpreting the results and preparing the manuscript. He was the corresponding author of the paper.

ABBREVIATIONS

AAS	α-aminoadipic semialdehyde
Ala or A	alanine
Arg or R	arginine
Asn or N	asparagine
Asp or D	aspartic acid
BSA	bovine serum albumin
CD	conjugated diene hydroperoxide
Cys or C	cysteine
DHP	dihydroxypyridine
DOPA	3,4-hydroxyphenylalanine
ESI	electrospray ionization
GC	gas chromatography
GGS	γ-glutamic semialdehyde
Gln or Q	glutamine
Glu or E	glutamic acid
Gly or G	glycine
His or H	histidine
HNE	4-hydroxy-2-nonenal
HPLC or LC	high-performance liquid chromatography
HPSEC	high-performance size exclusion chromatography
Ile or I	isoleucine
Leu or L	leucine
LOX	lipoxygenase
Lys or K	lysine
MDA	malondialdehyde
Met or M	methionine
MS	mass spectrometry
MS/MS	tandem mass spectrometry
O/W	oil-in-water
ONE	4-oxo-2-nonenal
Phe or F	phenylalanine
Pro or P	proline
PUFA	polyunsaturated fatty acid
PV	peroxide value
RT	retention time
SDS	sodium dodecyl sulfate
SPI	soy protein isolate
SPME	solid-phase microextraction
SSA	specific surface area
Thr or T	threonine
TMP	1,1,3,3-tetramethoxypropane
Trp or W	tryptophan
Tyr or Y	tyrosine
Val or V	valine
WPI	whey protein isolate
α-La	α-lactalbumin
β-Lg	β-lactoglobulin

1 INTRODUCTION

Oxidative degradation of protein and lipid components of food systems is one of the major concerns of research and industry due to its undesired consequences. Oxidation reactions involving proteins and lipids are known to cause loss of nutritional quality, deterioration of sensory attributes and unwanted textural modifications in foods (Kołakowska 2003; Lund and Baron 2010). Proliferation of new food products aiming to fulfil shifting interests in diet preferences requires investigations on the behavior of food components in new formulations and the underlying chemical reactions during the application of novel processing techniques. Accordingly, the phenomenon of oxidation continues to be center of focus for researchers and food producers alike.

While oxidation of lipids is an extensively researched topic, protein oxidation with respect to food research is gathering interest only relatively recently. Proteins are susceptible to actions of similar reactive species that initiate lipid oxidation. Furthermore, radical formation observed in both proteins and lipids leads to interactions between these molecules that affect the course of oxidation. Lipid oxidation products such as hydroperoxides and carbonyls are capable of inflicting oxidative damage on proteins (Hidalgo and Zamora 2002; Elias et al. 2005; Schaich 2005). The resulting modifications of proteins in foods can be manifested as loss of solubility, discoloration, decrease in flavor perception, polymerization, loss of digestibility and nutritional availability (Færgemand et al. 1998; Bertrand-Harb et al. 2002; Girón-Calle et al. 2003; Kühn et al. 2006; Utrera et al. 2014; Obando et al. 2015). Thus it is essential to monitor the course of these modifications in order to optimize solutions against oxidation in foods that contain proteins and lipids as major components.

Oxidative interactions of proteins and lipids may involve reactions at a molecular level marked as formation of adducts between proteins and oxidized lipid species some of which are highly reactive. Covalent binding of proteins with these reactive aldehydes leads to side-chain modifications and deterioration of protein functionality as well as propagation of further protein oxidation (Esterbauer et al. 1991; Wu et al. 2009b; Zhao et al. 2012). Moreover, these alterations show varying targets of attacks depending on the protein (Uchida and Stadtman 1992). Therefore, it is vital to characterize these varying pathways and identify the preferred sites of nucleophilic attacks.

As one of the most commonly utilized methods of delivery systems, emulsions are highly prone to oxidation due to their composition of lipids and proteins, especially when the latter are used as emulsifiers. Overall

progress of oxidation in emulsions depends strongly on the nature of the protein and lipid fractions. In particular, the roles of interfacial and continuous phase proteins on lipid oxidation have been studied with respect to their effects on stability of emulsions. However, the complex mechanism of oxidation affected by the intricacy of protein behavior under varying conditions makes it difficult to paint a full picture of the exact progress of oxidation in emulsions. These significant parameters that vary from system to system include structure of interfacial protein network, the concentration of unadsorbed proteins, the surface charge of interfacial membrane, droplet size, conformational state of the proteins, presence of pro-oxidants such as metals, availability of oxygen, and composition of proteins with respect to their radical scavenging side-chains (McClements and Decker 2000; Lethuaut et al. 2002; Villiere et al. 2005; Hu et al. 2005; Kiokias et al. 2006; Elias et al. 2008; Berton et al. 2011).

The interdependent relations of protein-lipid oxidation in emulsions also led to the investigations that sought to utilize the antioxidant activity of proteins both in their native and modified forms. Elias et al. (2005) attributed the antioxidant activity of continuous phase β-lactoglobulin to Cys and Trp residues, while Faraji et al. (2004) reported antioxidant activity of whey protein and soy protein isolates in emulsions due to their metal-chelating properties. Ries et al. (2010) found that increasing concentrations of unadsorbed proteins led to oxidatively more stable emulsions. Meanwhile, Berton-Carabin et al. (2013) studied possible antioxidant effects of heat-denatured proteins in emulsions but reported no improvements on oxidative stability. On the other hand, Kellerby et al. (2006a) investigated the same effect with enzymatically cross-linked proteins in their study that described no significant change in stability. However, Ma et al. (2012) reported reduced oxidation of lipids with enzyme-catalyzed cross-linking of proteins prior to emulsification. The complex nature of oxidative interactions between proteins and lipids renders these reactions remarkable topics of interest where there is still need for improvement for investigations in ascertaining the role of proteins and lipids in emulsions. Furthermore, increased attention paid to alternative protein sources points to a promising future for utilization of these proteins in emulsions.

Quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus caudatus*) and faba bean (*Vicia faba* L.) are some of the sources of plant proteins that have been attracting the attention of the consumers in the so-called Western world in recent years due to their gluten-free and rich protein content. Quinoa and amaranth are Andean grains with a protein composition made up mostly of albumins and globulins (Janssen et al. 2016). Protein extractability and solubility of both grains are higher at alkaline pH values (Elsohaimy et al. 2015; Fidantsi and Doxastakis 2001). Saponins in quinoa were found to increase the foaming and emulsification properties but reduce the emulsion

stability (Chauhan et al. 1999). Amaranth proteins were reported to have high foaming capacity and stability under acidic conditions while emulsifying activity was higher at pH 7.0 (Silva-Sánchez et al. 2004). Meanwhile, faba bean protein solubility was reported to increase below pH 4.0 and above pH 6.0 (Sosulski and McCurdy 1987). In addition, satisfying emulsifying and foaming properties of faba bean proteins were displayed in several studies (Makri et al. 2005; Karaca et al. 2011). Although some studies could be found on the functional properties of quinoa, amaranth, and faba bean proteins, the technological utilization of these protein sources are currently underexploited.

This dissertation presents an overview of the literature published on lipid and protein oxidation as well as their co-oxidative behavior at a molecular level and in emulsions systems. The purpose of the study is to investigate the interactions of proteins and lipids during oxidation. Objectives include characterization of adduct formations between oxidized lipids and peptides. Further aims covered assessment of the progress of oxidation in emulsion model systems and providing useful insight on the oxidative behavior of faba bean, quinoa and amaranth proteins and their effects on oxidative stability of these emulsion systems.

2 REVIEW OF THE LITERATURE

2.1 LIPID OXIDATION

Oxidation of food lipids includes a series of chemical reactions which take place during processing and/ or storage that lead to the deterioration of sensory quality, decrease in nutritional value, and undesired alterations of the textural properties of food products. These significant outcomes that determine the shelf-life have placed lipid oxidation in foods at the center of extensive studies for decades. Due to the complex system of reactions that initiate and perpetuate lipid degradation, oxidation still continues to be a topical issue for academy and industry.

Main factors affecting the onset, route, and rate of lipid oxidation are fatty acid composition (in particular, degree of unsaturation), oxygen pressure, contact area with oxygen, temperature, light, water activity, enzyme activity, and presence of pro- and anti-oxidants (Choe and Min 2006). Molecular oxygen in its ground state ($^3O_2$) is relatively unreactive towards nonradical lipid species. However, reactive oxygen species which form abundantly as a result of chemical, photochemical, and enzymatic reactions, are the main actors that catalyze lipid oxidation reactions. Reactive oxygen species (ROS) may include radical derivatives of oxygen such as hydroxyl, peroxyl, alkoxyl, and hydroperoxyl radicals as well as nonradical derivatives such as singlet oxygen, hydrogen peroxide, and ozone (Bartosz and Kołakowska 2011). Depending on the initiating factor, lipid oxidation follows several pathways which could be grouped as free radical-driven autoxidation, singlet oxygen-driven photosensitized oxidation (photooxidation), and enzymatic oxidation.

2.1.1 OXIDATION PATHWAYS

Main lipid oxidation pathways are usually elucidated under titles of autoxidation, photooxidation, and enzymatic oxidation (Kołakowska 2003).

Traditional explanation of free radical chain reactions of autoxidation mechanism involves three main stages: Initiation, propagation, and termination (Bateman 1954). At the initiation stage a hydrogen atom is abstracted from the lipid molecule to yield lipid alkyl radicals (L•), a reaction that is catalyzed by initiators such as existing free radicals, heat, exposure to light, and metals. Hydrogen atoms adjacent to the C=C double bond is removed easily, especially if the carbon atom is between two double bonds. Hence, the higher degree of unsaturation makes the fatty acid or the acylglycerol more prone to autoxidation. The double bond next to the carbon

atom radical, meanwhile, shifts to the adjacent more stable carbon and consequently transforms from *cis* to *trans* configuration. This configuration change leads to sole formation of conjugated oxidation products in the case of linoleic and linolenic acids (Choe and Min 2006).

Alkyl radicals formed at initiation stage then react with triplet oxygen ($^3O_2$) to yield peroxyl radicals (LOO•). During this propagation stage peroxyl radicals are the driving force of radical chain continuation via hydrogen abstraction from lipid molecules to form hydroperoxides (LOOH) and further alkyl radicals. These alkyl radicals in turn continue to react with oxygen and form more peroxyl radicals. The accumulation of hydroperoxides continue to a point where metals, UV light, and heat initiate the decomposition of lipid hyrdoperoxides to form highly reactive alkoxyl (LO•) and hydroxyl (HO•) radicals as well as further peroxyl radicals (Jeleń and Wąsowicz 2012). In addition to this classic description of the radical chain propagation stage, other reactions of peroxyl radicals that may take place prior to hydroperoxide formation are also known. These reactions of peroxyl radicals include rearrangement/ cyclization, addition on double bonds, disproportionation, β-scission, recombination, and electron transfer. Furthermore, alkoxyl radicals are also involved in these other reactions that propagate the radical chain besides hydrogen abstraction (Schaich 2005). These branching reactions that compete for peroxyl and alkoxyl radicals may yield different mixture of products depending on the oxidative conditions and affect the extent of oxidation. Therefore, the observed outcome of lipid oxidation is manifested as a result of a variety of complex propagation reactions that depend significantly on the oxidation system and other parameters.

Termination is the stage where nonradical and stable products of oxidation are formed through several mechanisms such as collision of radicals, scission of alkoxyl radicals, eliminations of hydroxyl (–OH) and hydroperoxyl (–OOH) groups from lipid hydroperoxides, and oxidative reactions with a nonlipid molecule (Schaich et al. 2013). One of the most elucidated pathways of termination product formation is the radical recombination where alkyl, peroxyl, and alkoxyl radicals undergo collision reactions to form alkanes, alcohols, ketones ethers, and peroxides (Jeleń and Wąsowicz 2012). On the other hand, scission reactions of alkoxyl radicals yield oxidation products that are responsible for off-flavors and off-odors of rancid lipids. Elimination of hydroxyl and hydroperoxyl groups from lipid hydroperoxides leads to formation of ketones as the major product. The preferred pathway of termination reactions depend on several factors including available oxygen, solvent of the medium, temperature, the ongoing propagation of radicals, and the presence of antioxidants and other nonlipid molecules (Labuza and Dugan Jr. 1971; Schaich et al. 2013). A general scheme of autoxidation mechanism is presented in Figure 1.

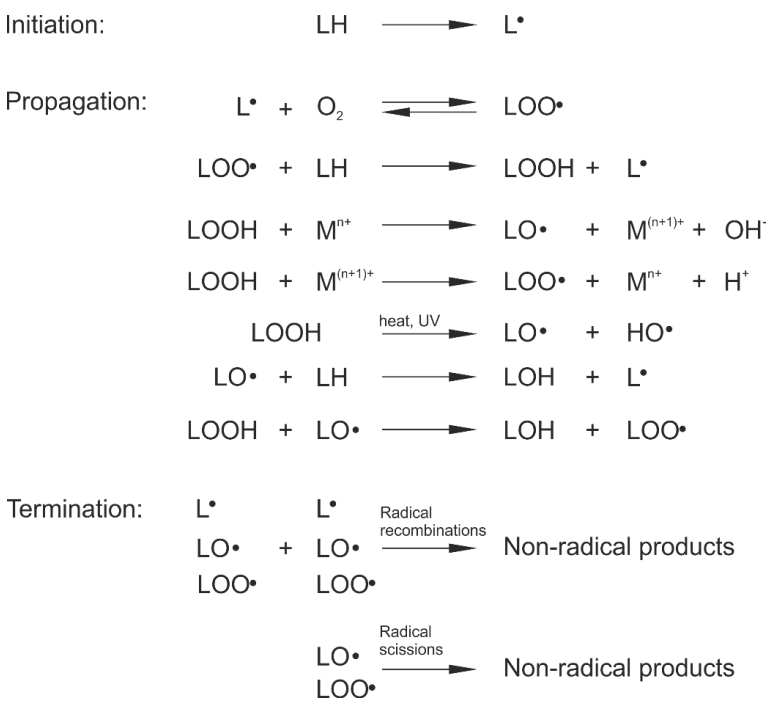

Figure 1 A general scheme for lipid autoxidation (L•: Alkyl radical; LOO•: Peroxyl radical; LO•: Alkoxyl radical; HO•: Hydroxyl radical; LOOH: Lipid hydroperoxide; M: Metal ion). Adapted from Jeleń and Wąsowicz (2012), Schaich et al. (2013)

Photooxidation refers to the initiation of lipid oxidation by light, photosensitizers, and oxygen. A photosensitizer such as chlorophyll, myoglobin, and riboflavin can absorb energy from light and reach its short-lived singlet-state (^{1}S) releasing energy to yield its excited triplet-state form (^{3}S). In this state, sensitizer is able to undergo photochemical reactions that ultimately initiate lipid oxidation in two major types of pathways. In Type I pathway, excited sensitizer reacts directly with lipid alkyls to form radicals which in turn react with ground-state triplet oxygen to initiate the previously explained radical chain-driven oxidation (Min and Boff 2002).

Type II pathway involves the generation of singlet oxygen ($^1O_2$) from the reaction of triplet-oxygen molecule ($^3O_2$) with ^{3}S molecule. As a result of this, sensitizer molecule returns to its ground singlet-state and continues to react with other triplet-oxygen molecules (Choe and Min 2006). In addition to photochemical reactions, singlet-oxygen can also be generated chemically and enzymatically (Krinsky 1977). Singlet-oxygen, unlike triplet-oxygen, is able to oxidize alkyl lipids to form hydroperoxides without the radical chain reaction and shows higher reactivity than triplet-oxygen (Min and Boff 2002). The major oxidative action of singlet-oxygen is the addition of the oxygen molecule to the carbon atom with the double bond in what is called

the “ene” reaction. This generates the hydroperoxide with double bond being shifted to allylic position and converted to *trans* isomer (Frankel 1991). As a result, unlike autoxidation, singlet-oxygen reactions with lipid molecules do not depend on the location of the double bond but only on the amount of double bonds. Consequently, the types of hydroperoxides formed from Type II pathway may differ from those originating from radical chain reactions. Singlet-oxygen oxidation can yield both conjugated and non-conjugated diene and triene hydroperoxides unlike triplet-oxygen reactions. Furthermore temperature does not play a relevant role on singlet-oxygen oxidation as high activation energy is not required for these reactions (Min and Boff 2002). Hydroperoxides formed in both types of oxidation are decomposed the same way but the variance of the hydroperoxides formed may result in different decomposition products and hence affect the observed outcome of lipid oxidation (Choe and Min 2006).

Enzymes that catalyze lipid oxidation in foods are present in various plant and animal cells, separated from their substrates and are inactive until they come into contact with lipid molecules upon processing. Enzymatic activity contributes to flavor formation in food systems by generating hydroperoxides directly from lipid molecules and molecular oxygen which further decompose into flavor compounds (Jeleń and Wąsowicz 2012).

Lipoxygenase (EC 1.13.11.12) is the predominant group of enzymes that initiates and determines the fate of lipid oxidation in food raw materials. Lipoxygenase (LOX) specifically targets polyunsaturated fatty acids (PUFA) with *cis,cis*-1,4-pentadiene moieties in the presence of molecular oxygen to produce hydroperoxides without the release of free radicals (Axelrod et al. 1981). The enzymatic reaction itself does not initiate radical formation but decomposition of hydroperoxides yields reactive peroxyl, alkoxyl, and hydroxyl radicals takes place under favorable conditions such as presence of pre-formed radicals, metals, light, and heat (Schaich 2005). Therefore, conditions that keep LOX activity under control in foods are important to hinder initiation of specific hydroperoxide formation which leads to flavor-active secondary oxidation products. The LOX pathway proceeds in a specific way depending on the fatty acid as the substrate, the LOX isozyme present in the food material, oxygen pressure, medium of the oxidation, and in particular, pH conditions preferred by the enzyme (Gardner 1988).

Another group of enzymes that is active in enzymatic oxidation pathway is hydroperoxide lyase (HPL). HPL acts on the hydroperoxides generated via LOX reactions catalyzing the cleavage of these molecules into corresponding aldehydes and oxo-acids. It is known that HPL prefers 9- and 13-hydroperoxides that are produced from linoleic and linolenic fatty acids (Kim and Grosch 1981). These specific cleavage products may further be enzymatically converted to their isomers and corresponding alcohols by the activity of isomerases and alcohol dehydrogenases, respectively (Grechkin

2002). Enzymatic pathway of oxidation in foods is responsible for many characteristic aroma compounds due to the volatile compounds formed as well as undesired off-flavors. In the case of formation of unwanted lipid oxidation products, processing and storage conditions need to be controlled in order to inhibit the specific activity of these enzymes.

2.1.2 OXIDATION PRODUCTS AND THEIR ANALYSIS

2.1.2.1 Primary products

The main intermediary products of lipid oxidation are hydroperoxides which can arise both directly via enzymatic pathway and attack of the radical species. Lipid radicals are generated readily through hydrogen abstraction from the carbon atoms that are next to the doubly-bound carbons in the allyl group which in turn yield the various corresponding hydroperoxides of the fatty acid. Depending on radical positional distribution within the chain, the resulting hydroperoxides may have *trans* and/or *cis* configuration of the double bond (Belitz et al. 2009).

In the case of oleic acid (18:1 Δ9) autoxidation, main hydroperoxides with respect to the location of hydroperoxide group were found to be a mixture of 8-, 9-, 10-, and 11-hydroperoxides. The pentadienyl radicals that form in PUFA such as linoleic (18:2 Δ9, 12) and linolenic (18:3 Δ9, 12, 15) acids lead to the occurrence of conjugated hydroperoxides. Linoleic acid oxidation produces the corresponding 9- and 13-hydroperoxides, while those generated from linolenic acid were reported to be a mixture of 9-, 12-, 13-, and 16-hydroperoxides among which 9- and 16-hydroperoxides were the dominating products (Frankel 1980; Chan et al. 1982). Mechanism of hydroperoxide formation from linoleic acid is illustrated in Figure 2.

Hydroperoxides as early indicators of oxidation have been measured in various methods in analyses of lipid oxidation. One of the most traditional methods employed in measurement of hydroperoxides is the determination of the peroxide value (PV) which refers to the milliequivalents of oxygen per kilogram of fat or oil. This iodometric method is based on the reaction of the hydroperoxide group with potassium iodide (KI) where iodide ions are oxidized and in turn iodine is released. Free iodine then can be titrated with sodium thiosulphate ($Na_2S_2O_3$) in the presence of a starch indicator and thus provides direct information on the amount of peroxides present. This method is sensitive to the amount of oxygen present especially if the released iodine levels are low (Pokorný et al. 2005). An alternative method to iodometry with an increased sensitivity that utilizes the reduction of hydroperoxide group is the ferric thiocyanate technique. The reaction of ferrous chloride with the hydroperoxide group leads to the oxidation of Fe^{2+} ions to Fe^{3+} ions. In the presence of ammonium thiocyanate, Fe^{3+} ions form the red-colored ferric

thiocyanate complex which can be detected spectrophotometrically at 500 nm (Kiokias et al. 2010).

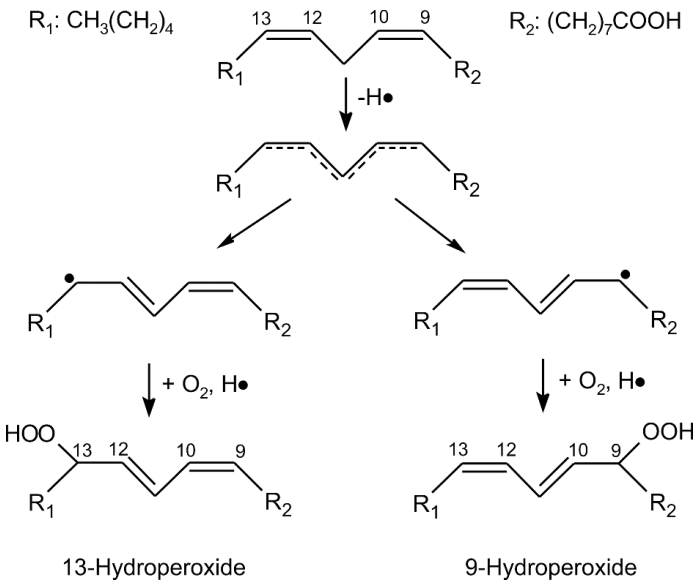

Figure 2 Formation of the major hydroperoxides from linoleic acid oxidation (Adapted from Min and Boff 2002).

Another method for monitoring primary lipid oxidation includes the spectrophotometric measurement of conjugated diene hydroperoxides. These hydroperoxides originate from PUFA especially with double bonds in 1,4-pentadiene structure. The corresponding conjugated hydroperoxide absorbs UV light strongly at 232-234 nm and the absorbance intensity is directly proportional to the concentration in the oxidized oil (White 1995). Thus the measurement of conjugated dienes in oil sources rich in linoleic acid provides insight into the progress of early lipid oxidation. The quantitation is based on the absorbance and molar absorptivity of linoleic acid. The advantages of this method include its lack of necessity for chemicals for color development and its rapid application. However, it is limited to the composition of the lipid molecule as per requirement of an abundance of PUFA with conjugated double bond structure. It was also reported that some compounds such as carbonyls with conjugated double bonds absorb UV light at a maxima of 245 nm (Pokorný et al. 2005). Therefore while monitoring progress of lipid oxidation, a multifaceted approach should be adopted that considers other aspects of the oxidation.

2.1.2.2 Secondary products

Secondary lipid oxidation products are stable, non-radical compounds that form during the decomposition of hydroperoxides and propagation stage some of which are volatile. The volatile products are molecules with lower molecular weight than the original lipid alkyl source which may contribute to the aroma of the food product and thusly are given utmost attention while some may be employed as indicators of rancidity. Secondary products may also include non-volatile monomeric, dimeric, oligomeric or polymeric molecules.

Generation of stable secondary products takes place mainly as a result of radical recombinations, reactions of alkoxyl radicals, and co-oxidation of non-lipid molecules. Radical recombinations which highly depend on temperature and oxygen conditions include reactions of alkyl, peroxyl, and alkoxyl radicals in vast possibilities of combinations that yield numerous products such as alkane polymers, alcohols, ketones, epoxides, peroxides and ethers. Among these, ketones and dialkyl peroxides were reported to be specifically arising as a result of recombination reactions (Schaich et al. 2013). Aldehydes as significant odor-active compounds are formed mainly through β-scission reactions of alkoxyl radicals. Alkoxyl radicals are first generated from hydroperoxides through hemolytic cleavage of the O–O bond and release a hydroxyl radical. From the resulting alkoxyl radical hemolytic cleavage of the C–C bonds on either side of the alkoxyl group yields an alkyl radical and aldehyde. Alkyl radicals released from this break down further react with alkyl lipids and other radicals present to generate a broad range of non-radical products (Frankel 1980). Main scission products originating from oleic, linoleic, and linolenic acids are presented in Table 1. Among the diverse oxidative breakdown products, some of the unsaturated aldehydes that arise from secondary oxidation products of PUFA are significant due to their high reactivity towards amino groups of proteins (Esterbauer et al. 1991). These include 4-hydroxynonenal, 4-oxo-nonenal, and malondialdehyde (MDA). MDA is formed through scission pathway of cyclic internal hydroperoxides that originate from oxidation of PUFA with at least three double bonds (Schaich et al. 2013). These compounds will be discussed later with respect to their reactions with proteins.

Traditional methods that determine lipid oxidation through monitoring of secondary oxidation products generally focus on aldehydes generated through scission pathways and carbonyls with volatile nature. One the most common parameters used in evaluating oxidative stability is *p*-anisidine value. The test of this value relies on the reaction of 2-alkenals and 2,4-alkadienals with *para*-anisidine reagent under acidic conditions to produce the yellow color that can absorb light at 350 nm. The measured absorbance value is used in calculating the *p*-anisidine value to be expressed as the 100 fold absorbance per g fat/ oil in 100 mL of solvent and *p*-anisidine mixture

(White 1995). Another traditional method that is based on aldehyde determination is 2-thiobarbituric acid (TBA) test. This method is based on the pink color complex formation between MDA and TBA reagent that absorbs light strongly at 532 nm (Kiokias et al 2010). However, it has been repeatedly reported that TBA color complex formation is not specific to MDA but also towards non-lipid molecules such as proteins and sugars, and therefore this method is also appropriately named 2-thiobarbituric acid reactive substances (TBARS) test (Bartosz and Kołakowska 2011; Papastergiadis et al. 2012). Another technique that employs spectrophotometry is the determination of total carbonyl value. Carbonyl measurement in this technique is achieved by the reaction of 2,4-dinitrophenylhydrazine with the carbonyls as catalyzed by trichloroacetic acid. Colorimetric measurement of the resulting complex produces maximum absorption at 430 nm for saturated aldehydes and at 460 nm for unsaturated aldehydes (Henick et al. 1954). However the technique is criticized due to undesired carbonyl formation during experiments as hydroperoxides decompose and the lack of specificity for only lipid-sourced carbonyls (White 1995).

Table 1 Main secondary autoxidation products of oleic, linoleic and linolenic acid.

Oleic acid	Linoleic acid	Linolenic acid
Heptanal	Hexanal	2,4-Heptadienal
Octanal	2-Heptenal	3-Hexenal
Nonanal	2-Octenal	2-Hexenal
Decanal	2,4-Decadienal	2-Pentenal
2-Decenal	3-Nonenal	3,5-Octadien-2-one
2-Undecenal	2-Nonenal	3,6-Nonadienal
	2,4-Nonadienal	
	2-Pentylfuran	

Compiled from Frankel (1982), Schaich (2005), Belitz et al. (2009).

In addition to the colorimetric tests, instrumental analyses that detect secondary oxidation products are also used extensively. A common technique to detect secondary oxidation products is gas chromatography (GC). GC methods take advantage of the volatility of numerous low-molecular-weight molecules to transfer them into gas phase and achieve chromatographic separation of individual compounds. GC sample introduction techniques may include direct injection as well as extracting the volatiles into a headspace such as in dynamic and static headspace analyses. A relatively new analyte introduction method is headspace solid-phase microextraction (SPME) and

is usually coupled with GC for separation and mass spectrometry (MS) for detection and identification. The principal of SPME is based on the adsorption of analytes from the sample headspace onto a fiber coated with a polymer film and subsequent desorption of volatiles into the GC injector. The type of fiber to be used for SPME analysis must be chosen according to the compounds of interest in the sample as the adsorption of volatiles of similar polarity will have the affinity for similar fibers. Common stationary phases of fibers include polydimethylosiloxane (PDMS), divinylbenzene (DVB), polyacrylate (PA), carboxen (CAR), and carbowax (CW) which can be used in combinations to increase the range of analytes adsorbed. The efficiency of SPME analyses depends on several parameters since the volatiles that are extracted from the sample to headspace depend on an equilibrium, as well as the equilibrium between headspace and fiber during adsorption. These parameters include film thickness, sample volume, extraction temperature, and time as well as desorption temperature and time (Wardencki et al. 2004). Analyses of volatile lipid oxidation products with SPME have been successfully carried out in emulsions (Beltran et al. 2005), rapeseed oil autoxidation (Jeleń et al. 2007), photosensitized linoleic acid oxidation (Lee and Min 2010), and spray-dried sunflower oil emulsions (Damerau et al. 2014) among other studies.

As mentioned before, not all secondary oxidation products are volatile or low-molecular-weight compounds that may contribute directly to the aroma of the food. Especially in heated oils and other lipids oxidized at high temperatures, dimeric, oligomeric, and other polymerized products are formed that contribute to the viscosity of the bulk oils and affect the nutritive value of lipids (Pokorný et al. 1976). Oxidation products with higher molecular weights can be analyzed through high-performance size exclusion chromatography (HPSEC) coupled with refractive index detector (RI) or evaporative light scattering detector (ELSD). HPSEC separates compounds according to their molecular size which is assumed to be proportional to their molecular weight in the case of lipid molecules (Marquez-Ruiz and Dobarganes 2005). Based on this, HPSEC is able to monitor the progress of lipid oxidation as oligomeric compounds are formed during storage.

2.2 PROTEIN OXIDATION

Research interests in oxidative stability of foods have been mainly dominated by lipid oxidation while protein oxidation has been mostly studied within biological systems or with respect to biological importance. However, as the consumer focus on protein-rich products and the awareness on the industrial utilization of proteins are increasing, significance of the

consequences of protein oxidation in food systems such as loss of nutritional value and undesired textural alterations is being realized more than before.

2.2.1 OXIDATION MECHANISM

Initiators of oxidative reactions in proteins include a similar selection of reactive species as in lipid oxidation. As such, these can originate from irradiation, oxygen, metal-catalyzed systems, peroxides, non-protein radicals, and free radicals. In particular reactive oxygen species such as hydroxyl (HO•), hydroperoxyl (HOO•), and superoxide anion ($O_2^{\bullet -}$) show strong tendency of initiating formation of protein radicals. Thus major pathways of oxidation involve formation of protein radicals which are instigated by hydrogen atom abstraction. The sites for the abstraction include the carbon atom at the α-position of amino acids, susceptible locations at amino acid side-chains, and polypeptide backbone (Stadtman and Levine 2003). In the presence of oxygen, these carbon-centered radicals (P•) rapidly generate peroxyl radicals (POO•) which may form hydroperoxide (POOH) derivatives with a suitable hydrogen donor present, or alkoxyl radicals (PO•) and consequently form hydroxyl (POH) derivatives (Stadtman 1993). Another significant mechanism of initiation of oxidation reactions is the formation of hydroxyl radicals via metal-catalyzed systems. The Fenton reaction involves the conversion of Fe^{2+} to Fe^{3+} while hydroxyl ions and hydroxyl radicals are formed from H_2O_2. Moreover, Fe^{2+} ions are also involved in breakdown of hydroperoxides to hydroxyl ions and reactive protein alkoxyl radicals (Davies et al. 1995). The main consequences of further reactions of radicals include peptide backbone cleavage, side-chain modifications of amino acid residues and dimerization through cross-link formations.

Peroxyl and alkoxyl radicals of main-chain α-carbons are known to be involved in backbone fragmentation in different pathways. Elimination of the hydroperoxyl radical group from peroxyl radicals leads to an intermediate imine formation which subsequently is hydrolyzed to corresponding amides and carbonyls. Furthermore, peroxyl radicals may also yield protein hydroperoxides via a hydrogen donor which converts the hydroperoxide to alkoxyl radicals. These alkoxyl and peroxyl radicals are in turn involved backbone cleavage. Polypeptide backbone cleavage was elucidated to take place via two pathways named α-amidation and diamide pathways (Garrison 1987; Stadtman and Levine 2003). In α-amidation pathway, backbone cleavage yields an amide derivative of the C-terminal amino acid and an α-keto-acyl derivative of the peptide on the N-terminal. On the other hand diamide pathway leads to formation of a diamide derivative of the new C-terminal amino acid residue and an isocyanate derivative of the new N-terminal amino acid residue (Figure 3). Meanwhile in the absence of oxygen

alkyl peptide radicals of α-carbons may undergo dimerization with cross-links forming between radical sites (Davies 2005).

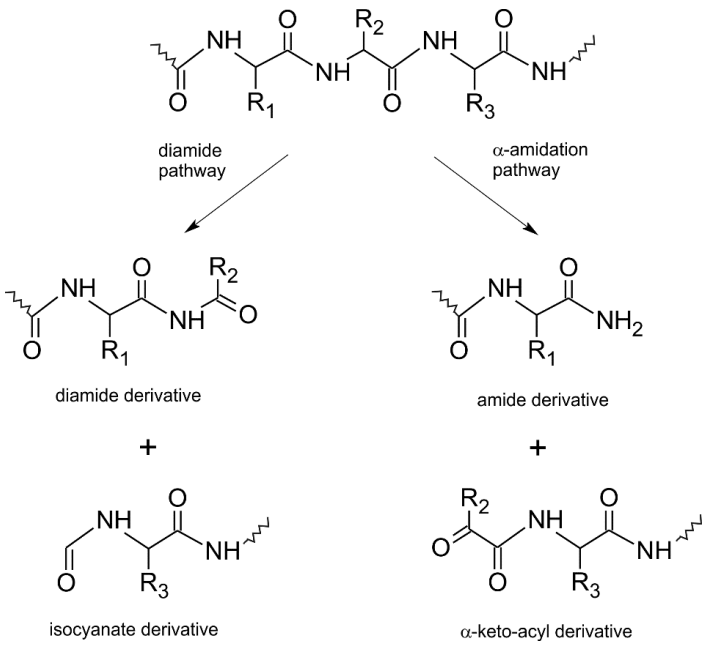

Figure 3 Oxidative backbone cleavage of polypeptides via α-amidation and diamide pathways (Adapted from Stadman and Levine 2003).

Side-chains of amino acid residues are also significant sites of radical attack within protein oxidation system. Consequences of reactions on the side-chains of amino acid residues include backbone fragmentation, cross-link formations leading to dimerization, and generation of unstable hydroperoxides, alcohols, and other derivatives of the residues. Some amino acid side-chains are more susceptible to oxidative attack than others as these may contain sites where hydrogen abstraction, oxygenation or an addition of hydroxyl group occur easier. Amino acids with side-chains most prone to modification are: Sulfur-containing amino acids cysteine (Cys) and methionine (Met); amino acids with aromatic side-chains tryptophan (Trp), tyrosine (Tyr), histidine (His), and phenylalanine (Phe); and among other amino acids lysine (Lys), arginine (Arg), proline (Pro), leucine (Leu), isoleucine (Ile), glycine (Gly), and valine (Val) (Garrison 1987; Stadtman 1993; Davies et al. 1999; Requena et al. 2001). A range of products generated via oxidative reactions of these side-chains are compiled in Table 2.

Table 2 Products of oxidative side-chain modifications in amino acid residues.

Amino acid	Main oxidation product
Cysteine	Cystine
	Sulfinic acid, Sulfonic acid
Methionine	Methionine sulfoxide, Methionine sulfone
Tryptophan	2-, 4-, 5-, 6-, and 7-Hydroxytryptophan
	N-formylkynurenine, kynurenine, kynurenic acid,
	3-hydroxy-kynurenine
Tyrosine	3,4-dihydroxyphenylalanine (DOPA)
	Dityrosine
Histidine	2-oxo-histidine
Phenylalanine	*ortho*-, *para*-Tyrosine, Tyrosine
Lysine	α-aminoadipic semialdehyde (AAS)
	3-, 4-, and 5-hydroxylysine
Arginine	γ-glutamic semialdehyde (GGS)
Proline	γ-glutamic semialdehyde (GGS)
	3- and 4-Hydroxyproline
Leucine	3- and 4-hydroxyleucine
Valine	3- and 4-hydroxyvaline
Threonine	2-amino-3-keto butyric acid

Compiled from Taborsky (1973), Garrison (1987), Stadtman (1993), Morin et al. (1998), Davies et al. (1999), Requena et al. (2001)

Amino acids Met and Cys are preferred sites of radical attack or hydrogen abstraction due to their sulfur-containing groups. Figure 4 summarizes the structures of oxidation products of Met and Cys residues. Cys oxidation occurs via hydrogen abstraction of the thiol group generating thiyl radicals which are easily oxygenated to yield the short-lived intermediate product sulfenic (R–SOH) acid. Further reactions of sulfenic acid with oxygen cause the formation of sulfinic (R–SOOH) and sulfonic acids ($R–SO_2OH$). Additionally, radicalization of sulfenic acid to thiyl radicals leads to formation of disulfide bridges and dimerization into cystine (Hawkins and Davies 2001, Rehder and Borges 2010). Side-chain of Met on the other hand contains a thioether group that is reversibly oxidized to form methionine sulfoxide (R'SOR) which is further oxidized to yield methionine sulfone ($R'SO_2R$), irreversibly (Vogt 1995). The high susceptibility of Met to oxidation has been studied in terms of its antioxidant activity against both intra-protein and non-protein molecules (Levine et al. 1999; Elias et al. 2005).

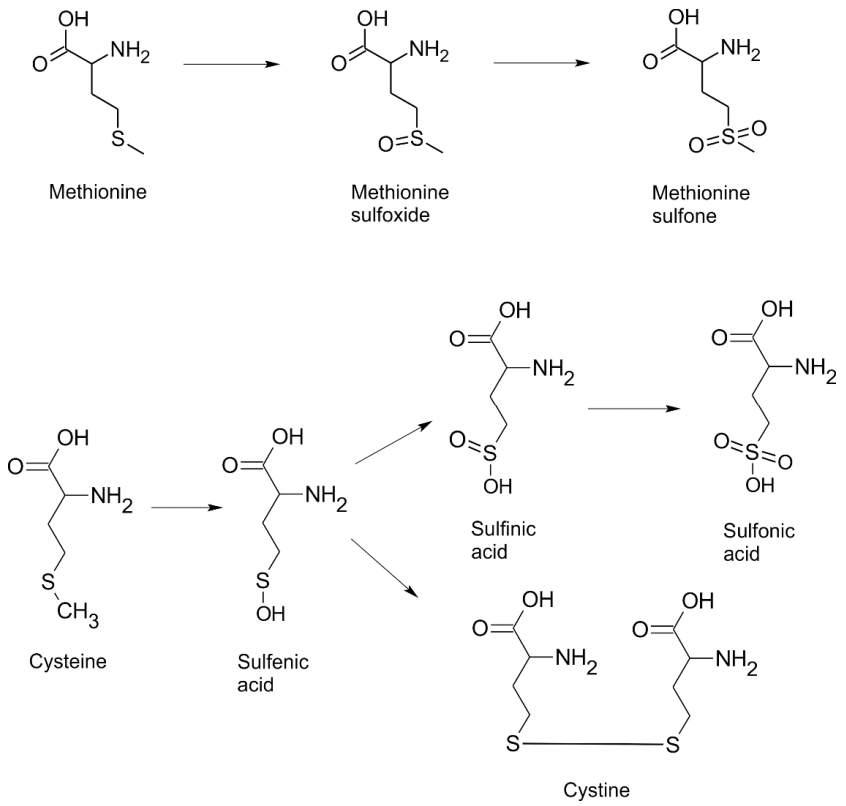

Figure 4 Oxidation products of methionine and cysteine side-chains. Adapted from Vogt (1995), Davies (2005), Rehder and Borges (2010).

Aromatic side-chains of amino acids are also highly prone to oxidative reactions. Main products of oxidative degradation of aromatic amino acid residues are presented in Figure 5. Reactions of the ring structure with the hydroxyl radicals usually involve the addition of the hydroxyl group. Oxidation of Tyr residues results in formation of hydroxylated radicals of the aromatic ring which further undergo disproportionation of two phenoxyl radicals in the absence of oxygen to yield 3,4-dihydroxyphenylalanine (DOPA). In the presence of oxygen, DOPA is generated per Tyr residue as a result of peroxyl radical formation from which hydroperoxyl group is rapidly eliminated (Gieseg et al. 1993; Davies 2005). The other outcome of Tyr oxidation is the formation of dityrosine which results from dimerization of Tyr radicals at the *ortho* position on the aromatic ring. Other than the hydroxyl radical attack, dityrosine may also arise as a result of UV irradiation, peroxidase-, and metal-catalyzed oxidation reactions (Huggins et al. 1993; Malencik and Anderson 2003). Oxidative degradation of Trp residues may take place as hydroxylated radical formation both on the phenyl and pyrrole moiety of the aromatic side-chain. Phenyl moiety oxidation

yields the isomers 2-, 4-, 5-, 6-, and 7-hydroxytryptophan (Maskos et al 1992). Hydroxyl radicals can also lead to addition on the pyrrole moiety, and in the presence of oxygen, generate peroxyl radicals. These radicals further react to cause cleavage of the heterocyclic ring and yield N-formylkynurenine, kynurenine, and 3-hydroxykynurenine as main products of Trp degradation. This consequent degradation results in loss of Trp fluorescence (Simat and Steinhart 1998; Davies et al. 1999). Meanwhile oxidative modification of His residues involves the formation of carbon-centered radicals on the imidazole ring which react with oxygen to yield peroxyl radicals. Subsequently, peroxyl radicals lead to formation of hydroperoxide intermediates from which the hydroxyl group is eliminated. The resulting major oxidation product is 2-oxo-histidine. This modification can take place in metal-catalyzed systems as well as singlet oxygen-driven oxidation systems (Uchida 2003). Main oxidation pathway for Phe residues also involves hydroxylation of the benzene ring in different positions and generate *ortho-*, *para-,* and *meta*-tyrosine (Garrison 1987).

Oxidative degradation of other amino acid residues with basic or aliphatic side-chains is initiated with carbon-centered radical formation via hydrogen abstraction especially due to reactive hydroxyl radicals. In the absence of oxygen these radicals may undergo dimerization which play a significant role in aggregation. In the presence of oxygen, however, peroxyl radicals are formed and yield a variety of end products including hydroperoxides and further radicals. In addition to the hydroxylated derivatives of these amino acids, decomposition of the hydroperoxides generates a range of radicals, alcohols, and carbonyl compounds. Carbonyl formation in these amino acids involve β-scission of the carbon-centered radical on the side-chain (Headlam and Davies 2004). Two of the major carbonyl products of radical-driven oxidation of Lys, Pro, and Arg are semialdehydes α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS). AAS originates from Lys residues while GGS is generates from Pro and Arg residues. In both pathways hydroxyl radicals initiate the side-chain radical formation followed by hydrolysis reactions that result in semialdehydes. From Lys, radical ammonium ion is released that leads to AAS formation, while GGS is formed as the guanidine group is hydrolyzed from Arg radical. In the case of Pro, a hydrogen abstraction from the heteroatom ring gives way to the formation of the semialdehyde (Requena et al. 2001; Akagawa et al. 2006).

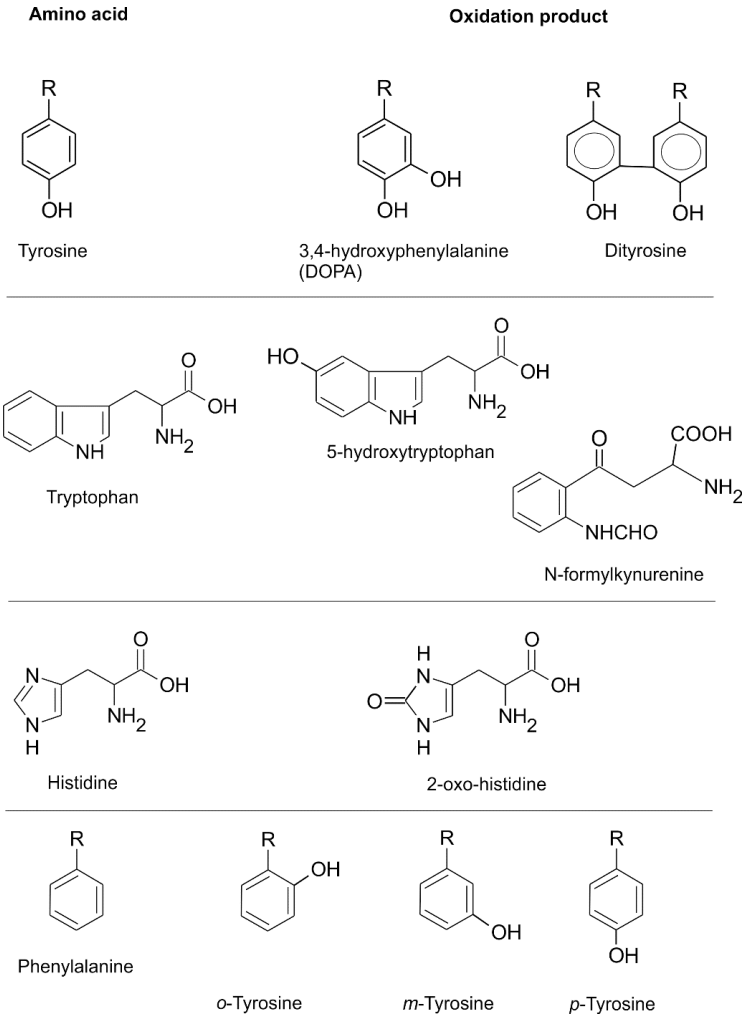

Figure 5 Main oxidation products of amino acid residues with aromatic side-chains. Adapted from Uchida and Kawakishi (1993), Stadtman and Levine (2003), Salminen et al. (2008).

2.2.2 ANALYSIS OF PROTEIN OXIDATION

Analyses of protein oxidation monitor the consequences of oxidative aggregation, polymerization, and fragmentation through detection of the side-chain modifications as well as measurement of nonspecific and specific carbonyls, and structural changes such as cross-linking.

Traditional methods of detecting oxidized proteins involve carbonyl assay. Carbonyls arising both via backbone fragmentation and side-chain degradation can be quantified spectrophotometrically as in lipid oxidation analysis through their reaction with 2,4-dinitrophenylhydrazine (DNPH).

DNPH method has also been optimized to allow the use of liquid chromatography with UV detectors (Levine et al. 1994; Headlam and Davies 2004). This traditional method was recently updated in a study published by Soglia et al. (2016) who targeted protein solubilization and unfolding prior to the reaction with DNPH. The modified method resulted in measurement of protein carbonyl content in three to fourfold that were otherwise under-quantified with the traditional method. Two specific carbonyl products AAS, GGS have also been employed in modern methods as major markers of protein oxidation. Requena et al. (2001) utilized GC-MS to quantify AAS and GGS following their reduction to 5-hydroxy-2-aminovaleric acid and 6-hydroxy-2-aminocaproic acid, respectively. Akagawa et al. (2006) on the other hand, derivatized the semialdehydes with a fluorescent agent and acid-hydrolyzed to monitor the hydrolysates with HPLC coupled with a fluorescent detector. Estévez et al. (2009) investigated the formation of AAS and GGS in α-lactalbumin (α-La), bovine serum albumin (BSA), and soy proteins using LC-MS/MS with electrospray ionization (ESI).

Another method of detection of protein oxidation utilizes the susceptibility of the sulfur containing side-chains of the amino acid residues. A traditional technique developed by Ellman (1959) relies on detecting the loss of thiol group by measuring the absorbance at 412 nm following the reaction with 5,5'-dithiobis(2-nitrobenzoic acid). The method was further improved (Thannhauser et al. 1983; Damodaran 1985) to analyze disulfide bonds which included the cleavage of the bonds by sodium sulfite and further specific reaction of free thiols with 2-nitro-5-thiosulfobenzoic acid. More recent improvements to the quantification of thiol and disulfides were implemented by Hansen et al. (2007) who utilized 4-4'-dithiodipyridine (4-DPS) as the thiol reagent and sodium borohydride as the reducing agent in a HPLC assay. This 4-DPS method was further optimized by Rysman et al. (2014) to detect protein oxidation in ground beef during storage at 4 °C.

Modifications of aromatic amino acids are also commonly used in analysis of oxidation. Among these, Trp residues are considered most prone to rapid oxidative degradation. Trp residues are known to exhibit strong fluorescent characteristics around 340 nm upon excitation at around 280 nm. Formation of oxidation products of Trp residues results in a directly correlated loss of native Trp fluorescence in proteins (Davies et al. 1987). Thus loss of native fluorescence has been employed to monitor oxidative degradation in proteins as a simple and sensitive method (Simat and Steinhart 1998; Viljanen et al. 2004; Dalsgaard et al. 2007; Salminen et al. 2008; Estevez et al. 2008). Furthermore, accurate identification of Trp oxidation products was also accomplished using LC-MS techniques (Finley et al. 1998; Domingues et al. 2003; Gracanin et al. 2009; Koivumäki et al. 2017). Dityrosine, as a significant product of Tyr dimerization, has also been monitored to detect oxidative modifications. Due to its absorbance and

intrinsic fluorescence characteristics, spectroscopic techniques and detectors coupled with chromatographic methods have been employed extensively (Huggins et al. 1993; Giulivi and Davies 2001; Dalsgaard et al. 2011; Scheidegger et al. 2013). Moreover, quantitative determination of dityrosine via labelled analytes has been achieved in milk powders (Fenaille et al. 2004a) and grain proteins (Nguyen et al. 2017) using isotope dilution LC-MS.

One of the major consequences of protein oxidation is polymerization. As mentioned before, cross-linking between amino acid residues or proteins can occur through disulfide bridges of Cys residues and dityrosine formation of Tyr radicals as well as between protein alkyl radicals. A straightforward method to detect these changes in molecular weight involves sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE provides visualization of the changes in molecular weight and can be used to monitor both oxidized protein as a whole (Davies and Delsignore 1987; Liu and Xiong 2000) and enzymatic digests (Ooizumi and Xiong 2006). Færgemand et al. (1998) also employed size exclusion chromatography to monitor enzymatic polymerization of whey proteins at 280 nm which is a specific wavelength for UV absorbance of aromatic amino acids.

2.3 PROTEIN-LIPID INTERACTIONS

Proteins and lipids are major components of various food systems and it would stand to reason that they would significantly influence each other within the complex mechanisms of oxidation. Appropriately it is relevant and necessary to deal with the oxidation in food systems with protein and lipid constituents as an interdependent set of interactions to obtain a more complete overview. Even though the earlier studies focusing on oxidative interactions of proteins and lipids were carried out in the field of biochemical and biomedical sciences, this section presents a review of the literature of these interactions within food sciences which include a range of investigations on food proteins and lipids.

Protein and lipid interactions proceed after the initiation of oxidation in a subsequent and/or simultaneous manner that involves transfer of radicals between protein and lipid species, reactions with lipid hydroperoxides, adduct formation in proteins induced by oxidized lipids and structural changes of proteins such as fragmentation and cross-linking.

2.3.1 RADICAL TRANSFER

Upon initiation of formation of radicals at the susceptible sites of protein and lipid molecules, these reactive species can influence the other's route of

oxidative pathway through radical transfer as an addition reaction or hydrogen abstraction. Early studies utilizing Electron Spin Resonance (ESR) technique provided evidence of radical transfer with a focus on pre-oxidized reactive lipid species as a source of radicals creating proteins radicals (Roubal 1970; Schaich and Karel 1975, 1976). Depending on the availability in the protein structure, these radical species can be generated via hydrogen abstraction from several locations such as sulfur groups, α-amino groups of side-chains, and α-carbon sites. Amino acid residues most prone to radical attack from reactive lipid species are Cys, Trp, Met, His, Lys, and Arg (Schaich 1980).

Even though the main focus on the co-oxidation pathways has traditionally been on lipid oxidation reactions preceding those of proteins, radical transfers at the early stages of oxidation may take place from proteins to lipids. Østdal et al. (2002) observed the formation of lipid oxidation products following reactions with BSA radicals. On the other hand depending on the oxidizing system and medium, oxidative modifications of proteins may also occur simultaneously (Hidalgo and Zamora 2002). Two of the most susceptible amino acid residues, Cys and Trp have been found to be degraded prior to lipid hydroperoxide formation (Elias et al. 2005; Salminen et al. 2010). Berton et al. (2012b) also reported in a study of oxidized oil-in-water-emulsion system with β-lactoglobulin (β-Lg), β-casein (β-Cn), and BSA that protein modifications commenced before lipid radical attack as measured via oxygen uptake, Trp fluorescence, and conjugated diene (CD) formation. Reactions of radicals formed in both proteins and lipids lead to further oxidative products and modifications as mentioned in earlier sections as well as continue to play a role in propagation of oxidation and radical formation in both components.

2.3.2 REACTIONS OF PROTEINS WITH OXIDIZED LIPIDS

Protein-lipid interactions under oxidative conditions also include reactions of amino acids, peptides, and proteins with lipid oxidation products such as hydroperoxides and aldehydes which result in various outcomes including accumulation of protein carbonyls, adduct formation, cross-linking, and fragmentation.

Lipid hydroperoxides are capable of inducing amino acid and protein degradation by directly reacting with them or via radicals and secondary products formed through decomposition. In reactions with amino acid residues that are more prone to hydrogen abstraction, lipid hydroperoxides may decompose generating alkoxyl and peroxyl radicals which propagate oxidation (Karel et al. 1975). Degradation of His residues with lipid hydroperoxides was reported by Yong and Karel (1978), while Hidalgo and Kinsella (1989) observed degradation of Trp residues and dimerization via

disulfide cross-links in β-Lg induced by one of the main linoleic acid hydroperoxide, 13-hydroperoxyoctadecadienoic acid (13-HPODE). Refsgaard et al. (2000) reported an increase in formation of protein-derived carbonyls in metal-catalyzed oxidation of BSA with 13-HPODE. Accumulation of protein carbonyls were also observed by Wu et al. (2009a) during the incubation of soy proteins with 13-HPODE in addition to loss of sulfhydryl groups and α-helix structure of the protein leading to cross-linking and aggregation. Other significant losses of amino acids were reflected in Trp, Met, Cys, Pro, Val, and Leu in a study by Lqari et al. (2003) where lupin seed globulins were incubated with 13-HPODE. Further degradation outcomes were polymerization and fragmentation in proteins.

Moreover, lipid hydroperoxides were also found to directly interact with proteins in addition reactions. In a model study, Gardner et al. (1977) reported of the adduct formation between Cys and 13-HPODE which suggests the sulfhydryl group of Cys as the main site of bonding between these molecules. Another adduct formation between 13-HPODE and Lys side-chain was identified by Kato et al. (1999) as N^{ε}-(hexanonyl)lysine in which terminal amino group of Lys side-chain is bound to the lipid hydroperoxide derivative through an amide bond.

On the other hand secondary oxidation products of lipids that are generated via hydroperoxide decomposition are also known to be highly reactive towards proteins. Among others, most significant of these secondary oxidation products include malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and 4-oxo-2-nonenal (ONE). These aldehydes can react with the nucleophilic sites on proteins to form adducts via Schiff base formation, Michael addition, and cyclization.

Compared to other aldehydes, alkanals are less reactive towards susceptible protein sites and require lack of competition from unsaturated aldehydes in adduct formation (Meynier et al. 2004). Hexanal was found to favor Schiff base formation with N-terminal Lys and Phe residues in insulin B chain (Fenaille et al. 2003, 2004b). In these studies, butanal and octanal also displayed higher tendency in alkylation and dialkylation of N-terminal amino acids rather than the ε-amino groups of Lys side-chain. Furthermore, Suyama and Adachi (1980) reported formation of pyridine structures in cyclized addition of multiple molecules of propanal to amino acids Leu and Gly.

MDA is one of the most reactive compounds which forms covalent adducts with amino acids. This reaction may adversely affect availability of essential amino acids as nutritional loss. Girón-Calle et al. (2003) reported that MDA-bound Lys residues were not metabolized and absorbed in the gut, thus leading to loss of this essential amino acid. Wu et al. (2009b) observed that MDA caused decrease in soy protein solubility and increased protein aggregation while number of disulfide bonds and sulfhydryl groups declined

which was attributed to MDA-induced covalent bond formation between proteins. Chen et al. (2013) confirmed the polymerization and loss of solubility in soy protein isolate (SPI) modified by MDA, while they also found that *in vitro* digestion of SPI was hindered due to these MDA-induced alterations. Meanwhile, Adams et al. (2008) reported protein browning of casein modified by MDA.

MDA reactions with amino acids occur mainly via Schiff base formations with amine groups at amino acid side-chains and N-terminus amino groups of peptides mainly in a 3-amino-2-propenal derivative structure. Lys side-chains at the ε-amino group are known to be one of the main sites of Schiff base adducts (Uchida et al. 1997). Other sites prone to MDA modification include His imidazole moiety, Cys thiol group, and side-chain amino groups of Arg, Asn, and Gln (Esterbauer et al. 1991; Slatter et al. 1999, 2004; Zhao et al. 2012). In reactions of amino acid residues with excess amounts of MDA and presence of other aldehydes such as formaldehyde or acetaldehyde, two molecules of MDA may form the 1-substituted-4-methyl-1,4-subtituted dihydropyridine-3,5-dicarbeldehyde structure. This dihydropyridine (DHP)-type unit is generated as a secondary condensation product of Schiff bases (Kikugawa et al. 1984). The significant characteristic of this type of adduct is its strong fluorescence emission intensity at around 470 nm when excited at around 400 nm (Kikugawa et al. 1985). The two additional carbonyl groups on the DHP-type ring structure may lead to further reactions that include Schiff base formations and thus cross-linking (Slatter et al. 1998). Additionally, Itakura et al. (1996) identified another fluorescent compound in a study where a model Lys-containing peptide was treated with MDA. This compound contains a pyridinium ring bound to the DHP-type structure at C-4 position and is another example of MDA-induced cross-link formation (Uchida et al. 1997). MDA is capable of producing further cross-links between proteins via Schiff base adduct formation of two amino groups with one molecule of MDA in a 3-amino-1-propene structure (Kikugawa et al. 1985). In addition to this structure a cross-link between Arg and Lys residues of BSA incubated with MDA was also identified by Slatter et al. (2004) where Lys-MDA adduct reacts with Arg residues and Arg side-chain undergoes cyclization into an imidazole ring formation. Some of the MDA-induced modifications of Lys, Arg, and His are presented in Figure 6.

Unsaturated structures of HNE and ONE also make it possible for protein cross-links to arise in reactions where both Schiff bases and Michael addition compounds are produced. Itakura et al. (1998) elucidated the formation of a fluorescent compound that underwent cyclization through Schiff base and Michael additions of two Lys residues and one HNE molecule which also indicated a cross-link. This type of HNE-mediated cross-link was also reported by Carini et al. (2003) between an ε-amino group of Lys side-chain and the imidazole ring of His residue. Additionally, Zhang et al. (2003)

detected ONE-induced pyrrole-type cross-links between Lys residues via Michael addition. Furthermore, HNE and ONE are also involved in Strecker-type degradation of amino acids in a similar manner that α-dicarbonyl compounds in Maillard reaction systems due to their structural similarities. Formation of Strecker aldehydes in reactions follows the formation of an imine intermediate through hydrolysis and decarboxylation reactions of amino acids with α,β-unsaturated lipid aldehydes (Hidalgo et al. 2005). In several studies, Strecker-type degradation of amino acids with HNE, ONE, and other unsaturated lipid oxidation aldehydes were reported to yield Strecker aldehydes which are known to be aroma compounds, 2-alkyfurans, and pyrrole derivatives that contribute to polymerization and non-enzymatic browning (Adams et al. 2011a, 2011b; Zamora et al. 2013).

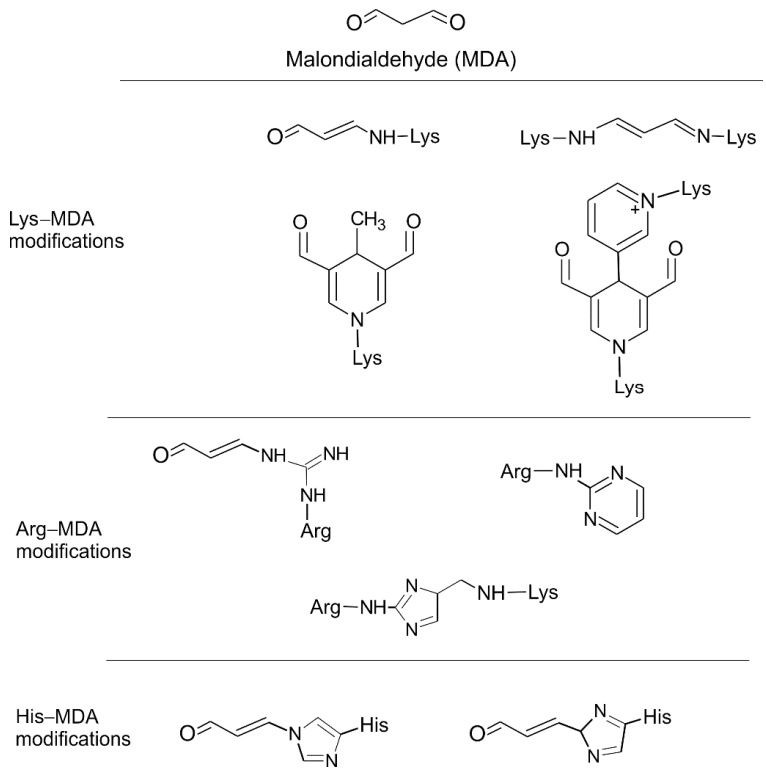

Figure 6 MDA-induced modifications of amino acids lysine, arginine and histidine. Adapted from Itakura et al. (1996), Uchida et al. (1997), Slatter et al. (2004), Adams et al. (2008), Zhao et al. (2012).

These specific modifications and characteristics that result from interactions of lipid oxidation aldehydes with proteins and amino acids enable development of analytical techniques to detect protein-lipid

interactions through utilization of the resulting structures as markers. A large majority of cited literature included the use of fluorescence detection and liquid chromatographic methods as well as tandem mass spectrometry to characterize and identify the interaction products.

2.3.3 CO-OXIDATION IN EMULSIONS

Oxidative interactions of proteins and lipids elucidated in earlier sections yield significant outcomes in food systems. As important components of foods oxidative degradation of these biomolecules leads to loss of nutritional value, alteration of textural properties, and undesired sensory attributes. Therefore, chemical modifications of proteins and lipids under oxidative conditions require accurate monitoring and assessment. Especially with growing attention given to products that contain proteins originating from plants as an alternative to meat and dairy sources, protein and lipid interactions gain significance in terms of oxidative stability. Food proteins and lipids have been studied within the complex and diverse pathways of oxidation with respect to the consequences of these interactions on food properties. This section reviews those studies with a focus on emulsions containing proteins as a major food system where co-oxidation has been frequently observed.

2.3.3.1 General information

Food emulsions are multi-phased colloidal systems where two immiscible liquids form a dispersion of one liquid in another in the form of droplets. Emulsions that contain oil droplets dispersed in the continuous water phase are named oil-in-water (O/W) emulsions. In these types of emulsions, oil droplets are kinetically stabilized with an interfacial layer with the help of emulsifiers. A simple representation of O/W emulsions is depicted in Figure 7.

Thermodynamically unstable nature of emulsions where the two immiscible liquids favor the separation of phases requires the utilization of emulsifiers. These are surface active compounds that decrease the surface tension between the oil and water phases and prevent the aggregation of the oil droplets via steric (where a physical barrier keeps the neighboring droplets from aggregating due to the molecular size of the surfactant) or electrostatic (where a charged interfacial layer formed around droplets repel each other) repulsion (McClements 2016). The stabilizing effect is manifested by forming layer around the freshly produced oil droplets as a result of the homogenization process (Coupland and McClements 1996). Two main mechanisms are observed during the breakdown of an emulsion, namely flocculation and coalescence. Flocculation is the reduction of the distance between two droplets due to the decrease in the strength of repulsive forces.

During this process droplets aggregate within a proximity of equilibrium distance. In the case of coalescence the interfacial barrier between the droplets are broken and the aggregation occurs where these droplets are physically incorporated into larger ones (McClements 2016). In food industry, low molecular-weight surfactants, phospholipids, and proteins are commonly utilized for the purpose of stabilizing emulsions. Proteins adsorbed at the oil-water interface achieve this stabilization due to their amphiphilic nature. Additionally, excess proteins that are not adsorbed at the interface are also be found in the continuous aqueous phase. Expectedly, presence of proteins play an important part on the overall oxidative stability, rate, and route of oxidative reactions in an interdependent manner with lipids.

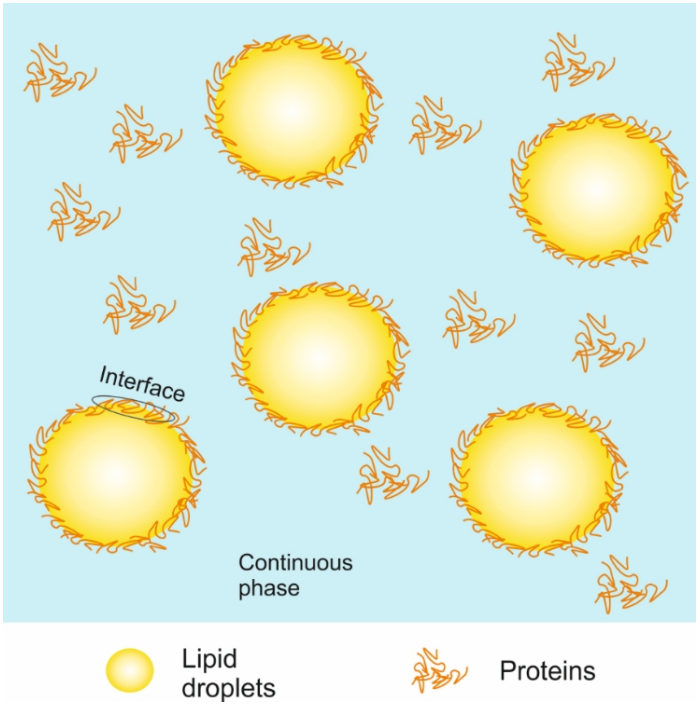

Figure 7 A representation of oil-in-water emulsions (not to scale with respect to molecules).

The effects of proteins on emulsion oxidation depend on several factors such as pH, ionic strength, and type of the protein. In terms of oxidative stability of lipids in emulsions, proteins were found to exert a certain antioxidant behavior due to the radical scavenging activity of Cys and Tyr residues and metal chelation (McClements and Decker 2000). Furthermore, antioxidant activity can be manifested by hindering propagation of oxidation

via previously mentioned interactions between proteins and lipids that yield non-radical unreactive species such as lipid hydroperoxides and bound aldehydes (Elias et al. 2008). The structure of the emulsifying protein determines the interfacial membrane thickness as well as the amino acid composition and surface exposure of certain amino acids that are prone to oxidative modifications such as polymerization (Dickinson 1998). Hu et al. (2003a) compared the effect of different proteins on oxidative stability of corn oil-in-water emulsions and found that casein was more effective against lipid oxidation followed by whey protein isolate (WPI) and SPI. Several studies attributed this effectiveness to casein's ability to form thicker interfacial layers, metal-chelating efficiency, and radical scavenging activity (Fang and Dalgleish 1993; Villiere et al. 2005; Clausen et al. 2009). There are various antioxidant studies that investigate the roles of interfacial and continuous phase proteins in emulsions. Lipid hydroperoxides formed upon oxygen uptake are more polar in nature than the lipid molecules they are derived from and hence tend to migrate to the interfacial area where they are more likely to interact with pro-oxidant metals that initiate their decomposition to free radicals (Hu et al. 2005). Thus, it makes the interfacial region a significant location where oxidative reactions take place. The behavior of proteins during oxidation at the oil-water interface may depend on several parameters such as interfacial film thickness, droplet size, pH, net electrical charge of the interface, and the concentration of continuous phase proteins.

2.3.3.2 Influence of interfacial layer on oxidation

Contribution of the interfacial layer in emulsions in hindering lipid oxidation had been investigated to observe if a thicker protein network at the interface would provide a barrier against pro-oxidants (Hu et al. 2003a; Kiokias et al. 2006; Berton et al. 2011). However, the effect of delayed lipid oxidation in these studies could not be attributed specifically to the effect of the proteins forming the thickest film at the interface since other factors such as metal chelating and radical scavenging activity of aqueous proteins could also be involved in the antioxidant behavior. This proposed effect of proteins acting as a barrier against pro-oxidants has also been studied involving alterations exerted on proteins to promote cross-linking to form a denser layer. Berton-Carabin et al. (2013) found that no substantial increase in oxidative stability was observed in emulsions stabilized with heat-denatured proteins. In another study, Kellerby et al. (2006a) reported that transglutaminase-catalyzed cross-linking of casein post-homogenization did not improve the oxidative stability of emulsions compared to the untreated samples. However, Ma et al. (2012) found that transglutaminase cross-linked sodium caseinate prior to emulsification provided better physical stability of the interfacial layer and enhanced protection against lipid oxidation.

Furthermore, Tikekar et al. (2011) reported that cross-linked WPI did not have a barrier effect against oxygen diffusion across the interface into dispersed lipid phase. Review of these studies shows that although the thickness of protein interfacial membrane may have a role in protecting lipids against pro-oxidants such as metals, a thicker network of proteins is most likely to be ineffective against oxygen or free radical transport through the interface due to the heterogeneous structure of adsorbed proteins stabilizing the lipid droplets. Moreover it should be kept in mind that oxidized proteins located at the interface may be involved in propagation of lipid oxidation via free radical transfer reactions.

Droplet size affects the rate of oxidation with respect to the surface area of oil-water interface in the emulsion. Smaller droplet size indicates a higher surface area for the access of pro-oxidants and oxygen to lipid molecules, a higher amount of fatty acids closer to the interface and an increased amount of adsorbed proteins around droplets. Accordingly, several studies have considered that an increased surface area of oil droplets would increase oxidative reactions. Gohtani et al. (1999) measured higher PV in docosahexaenoic acid (C22:6) emulsions with smaller droplets than larger droplets and attributed this to higher surface area for oxidative reactions. Lee et al. (2011) postulated that higher specific surface area was one of the reasons that they found lower oxidative stability in nanoemulsions compared to conventional emulsions. Lethuaut et al. (2002) observed a more advanced oxidation of lipids with higher surface area in BSA-stabilized sunflower oil emulsions. However, they found a lack of consistence between the magnitude of droplet size decrease and the rates of conjugated diene formation and oxygen uptake. It was suggested that the difference arose because the increased oxidation rate may have been counterbalanced by the antioxidant characteristics of interfacial BSA. Ries et al. (2010) also suggested that antioxidant activity of proteins in increased concentrations have a strong effect in countering lipid oxidation even in emulsions with smaller droplets and higher oil-water surface areas.

Another important aspect of interfacial layer in progress of the oxidation is the net charge around oil droplets. Since interfacial layer is a vital location where protein-lipid oxidation reactions take place, it was assumed that a cationic protein membrane would act as a repellent towards metal cations from aqueous phase and increase lipid oxidative stability. Kellerby et al. (2006b) conducted protein-stabilized emulsion experiments at pH levels lower than protein isoelectric point (pI) in order to study this effect of protective functions of positively charged interfacial layer. The higher oxidative stability of lipids was contributed to this repelling effect. However, several studies found that the magnitude of the protein membrane net charge was not directly correlated to protection of lipid droplets against oxidation. Hu et al. (2003b) assessed the oxidative stability in emulsions prepared with

β-Lg, α-La, sweet whey, and WPI. Even though sweet whey emulsion droplets had the lowest charge density, this emulsion was oxidatively one of the most stable. Similarly in another study, Hu et al. (2003a) pointed out that although zeta potential of droplets in WPI-stabilized emulsions was the highest among casein- and SPI-stabilized emulsions, it did not correlate with the oxidative stability. Berton et al. (2011) also found that among a number of emulsifiers including proteins and nonionic sufactants, emulsifiers with the lowest zeta potential did not necessarily provide the best protection against lipid oxidation. Thus, there seems to be a lack of overall correlation between the magnitude of the interfacial membrane net charge and protection against oxidation which points to other factors influencing the antioxidant behavior of proteins.

2.3.3.3 Role of continuous phase proteins

Continuous phase (unadsorbed) proteins are modified via oxygen- and metal-derived oxidation that has a profound effect on the course of lipid oxidation. Faraji et al. (2004) compared the oxidative stability of menhaden O/W emulsions prepared with SPI, WPI, and sodium caseinate with those from which the unadsorbed proteins were removed. They found that lipid oxidation was not as advanced when unadsorbed proteins were present which was attributed to the metal-chelating and radical scavenging activities of continuous phase proteins. The latter activity was due to the ability of Cys sulfhydryl groups to react with free radicals. Elias et al. (2005) later confirmed this antioxidant behavior of unadsorbed proteins via monitoring lipid hydroperoxides, TBARS, free sulfhydryl groups, and Trp degradation. They reported that free sulfhydryl and Trp degradation in continuous phase occurred before detection of lipid hydroperoxides and TBARS, thus delayed the lipid oxidation. In a later study, Elias et al. (2006) followed the oxidation of Tyr, Met, and Phe residues of β-Lg chymotryptic hydrolysates and found that when dispersed in continuous phase, these hydrolysates were better in inhibiting lipid oxidation while Tyr and Met residues themselves were oxidized extensively. Cheng et al. (2010) reached the same conclusion when potato protein hydrolysates, ~85% of which were dispersed in the aqueous phase, improved the oxidative stability of soy bean O/W emulsions due to radical scavenging and metal-chelating activities of these proteins. Berton et al. (2012b) reported that unadsorbed proteins delayed the onset of lipid oxidation most likely due to their antioxidant activities during the initial stages. However, once the propagation stage was underway, interfacial proteins exhibited higher levels of oxidative modifications due to interactions with lipid-derived radicals. These studies confirm that the role of continuous phase proteins seems to be a significant one with respect to the progress of oxidation in emulsions.

In general, more attention has been paid to the consequences of lipid oxidation in emulsions due to formation of aroma-active volatiles and loss of nutritional quality. Meawhile outcomes of protein modification in emulsions are only recently garnering interest. Kong et al. (2013) observed a negative effect of WPI oxidation on solubility and gelation while low levels of oxidation increased the emulsifying activity and foaming properties. Oxidation of whey proteins was found to lead to a decrease in elasticity at the oil-water interface due to formation of aggregates which lacked the ability to form an interconnected network (Berton-Carabin et al. 2016) while coalescence stability was decreased upon protein oxidation (Muijlwijk et al. 2017). Furthermore, *in vitro* digestibility of autoxidized whey proteins and photo-oxidized caseins was reduced in PUFA-enriched O/W emulsions compared to control protein solutions without oil (Obando et al. 2015). Therefore, in addition to sensory deterioration, oxidation of proteins is also involved in the undesired alterations exerted on the structural integrity of emulsion systems.

3 AIMS OF THE STUDY

The purpose of the study was to elucidate the degradation of proteins and lipids within oxidative system, and to characterize the consecutive modifications resulting from the oxidative interactions between food proteins and lipids in model investigations.

Details of the objectives were:

- To characterize the interactions between tryptic β-lactoglobulin peptides and lipid oxidation product malondialdehyde using liquid chromatography–tandem mass spectrometry technique in order to identify the newly formed adducts (I).

- To investigate the course of oxidative degradation of lipids and water-soluble quinoa and amaranth proteins in oil-in-water emulsions prepared with a high-pressure homogenizer and its effects on the physical stability (II).

- To assess the effects of microwave and conventional thermal treatments on the course of overall oxidation in oil-in-water emulsions stabilized by proteins extracted from faba beans and to characterize the oxidative behavior of adsorbed and unadsorbed proteins with respect to lipid oxidation (III).

4 MATERIALS AND METHODS

4.1 MATERIALS (I-III)

Purified and lyophilized bovine milk β-Lactoglobulin (β-Lg) and 1,1,3,3-tetramethoxypropane (TMP) used in the synthesis of malondialdehyde (MDA) in study I were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus caudatus*) grains used in study II were of commercial variety and imported from South America by Aduki Ltd. (Finland). Faba beans utilized in study III were grown at Viikki Experimental Farm of University of Helsinki and belonged the "Kontu" cultivar (Lizarazo et al. 2014). Rapeseed oil (Keiju Rypsiöljy, Bunge Finland Ltd., Raisio, Finland) used in emulsion-making (II-III) was purchased from a local store.

Trypsin (Sequencing grade, modified) was purchased from Promega Corp. (Madison, WI, USA). Soy Protein Isolate (Supro® EX 45 IP) was acquired from Solae™, LLC (St. Luois, MO, USA). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) (≥99%, GC), linoleic acid (≥99%), Tween®20, 2-propanol (CHROMASOLV®, for HPLC, 99.9%), heptane (CHROMASOLV®, for HPLC, ≥99%), ammonium bicarbonate (NH_4HCO_3), acetonitrile (HPLC, reagent grade), and 1,4-dioxane (≥99.5%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Soy milk with vanilla flavor (Alpro C.V.A., Wevelgem, Belgium) to be used as an in-house reference for SPME-GC-MS method was purchased from a local store. Tocopherol standards (α-, β-, γ-, δ-), aluminum oxide (Al_2O_3, 90 active neutral, activity stage I, for column chromatography, 0.063-0.200 mm, 70-230 mesh ASTM), sodium hydroxide pellets (NaOH), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), hydrochloric acid (HCl), and sodium azide (NaN_3) were acquired from Merck KGaA (Darmstadt, Germany). Water used in all studies was purified via Milli-Q equipment (Millipore Corp., Bedford, MA, USA).

4.2 ENZYMATIC DIGESTION OF β-Lg AND FRACTIONATION OF PEPTIDES (I)

Enzymatic digestion of β-Lg by modified trypsin and fractionation of the tryptic peptides were carried out according to the method described by Koivumäki et al. (2012). Modified trypsin (suspended in 50 mM acetic acid) was added to the protein solution, where β-Lg was dissolved in 50 mM ammonium bicarbonate buffer, with a ratio of 1:250 (protease: protein,

w/w). The digestion was carried out overnight at 37 °C. Enzyme activity was stopped the next day by placing the digest at the freezer (−20 °C).

Chromatographic separation, isolation, and fractionation of β-Lg peptides were performed using reversed phase preparative LC-MS, utilizing the auto-sampler collection with triggers set for the corresponding *m/z* values of the theoretical peptides and analyte retention times. The semi-preparative column used in chromatographic separation was Waters XBridgeTM Prep BEH130 (C18, particle size: 5 µm, 10 x 250 mm). UV absorbance detection range of 190-400 nm and the *m/z* value range of 500-2000 were set for Prep-LC-MS runs. Mobile phase used for the chromatographic separation consisted of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid acetonitrile.

The three tryptic β-Lg peptides selected for the study were: ALPMHIR (Ala158–Arg164, *m/z* 838), LIVTQTMK (Leu17–Lys24, *m/z* 934), and VLVLDTDYK (Val108–Lys127, *m/z* 1066). Auto-collected peptide fractions were placed at −20 °C until further use. Another tryptic β-Lg peptide, TPEVDDEALEK (Thr141–Lys151, *m/z* 1246) was also isolated, fractionated and stored in 10 mM ammonium bicarbonate buffer (pH 7.3-7.4) at −20 °C to be used as an in-house reference in analyses of the oxidation samples. Peptide fractions from four different Prep-LC-MS runs were pooled and solvents were evaporated with a rotary evaporator prior to oxidation experiment.

4.3 TREATMENT OF RAW MATERIALS AND PROTEIN EXTRACTION (II-III)

Saponins were removed from quinoa and amaranth grains (II) prior to milling by washing the grains under running cold water (~10 °C) until no more foaming was observed which was followed by air-drying. Milling was carried out using an ultra-centrifugal mill (Retsch ZM 200, Haan, Germany) at a speed of 10000 min^{-1} with sieve pore size of 0.5 mm. Total protein contents of the flour samples were measured according to Kjeldahl method. Heptane was used in defatting procedure of the flours prior to protein extraction. Samples were then centrifuged and supernatant was discarded. Residual heptane was evaporated in a vacuum oven at room temperature. Defatted flours were suspended in water (10% w/v) and pH was adjusted to 9.0 using 2 N NaOH. Suspensions were left stirring at room temperature for 2 h and stored at 4 °C overnight. Afterwards, samples were centrifuged at 9000 *g* for 20 min at 4 °C. Supernatant was filtered and pH was adjusted to 5.0 with 1 N HCl at 4 °C to precipitate the proteins. Samples were then centrifuged at 9000 *g* for 20 min, the precipitate was suspended in water and mixed using an Ultra-Turrax® T25 homogenizer (IKA®-Werke GmbH & Co. KG, Germany). Solutions were neutralized using 1 N NaOH and freeze-dried

to obtain the protein extracts which were later suspended in water. Samples were taken for protein content measurement before freeze-drying.

Conventional thermal and microwave treatments of faba beans (III) were carried out as reported by Jiang and others (2016). Conventional thermal treatment referred to heating faba beans with an air oven at 170 °C for 30 min. In order to avoid excessive water evaporation, the beans were packed in a sealed glass bottle during heating in triplicates. The microwave treatment was conducted by heating 200 g of faba beans with a microwave oven (microwave frequency 2450 MHz, Whirlpool JT-379, USA) at 950 watts for 1.5 min in five replicates. The treatments caused a moisture content decrease from 11.8% to around 10% in MWT and around 10.5% in CTT samples. After the treatments, beans were dehulled with a stone mill and ground with a high speed rotor ultra-centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch, Germany, sieve pore size 0.5 mm) into fine flour.

Flour samples of untreated (UT), microwave-treated (MWT), and conventional thermal-treated (CTT) faba beans were mixed with water with a ratio of 1:7 (w/v) and left to shake for one hour at room temperature in an oscillator (Grant OLS200, Grant Instruments, UK) at a speed of ~150 strokes/ min. Mixture was then centrifuged for 20 min at 15200 *g* at ~10 °C. Afterwards, supernatant was collected and filtered.

Protein contents of the extracts in studies II and III were determined spectrophotometrically using a protein assay kit (Bio-Rad *DC*™ Protein Assay, CA, USA) while BSA was used to obtain a standard curve.

4.4 EMULSION PREPARATION (II-III)

Rapeseed oil used was stripped of its tocopherols prior to emulsion preparation according to the method described by Lampi et al. (1999) with modifications. A glass column (51 cm x 2.9 cm i.d.) was packed with 180 g activated aluminum oxide and conditioned with heptane. Later, 100 g oil dissolved in 100 mL heptane was eluted to strip the oil of tocopherols, pro-oxidants, and trace metals. Purified oil-in-heptane was then stored at −20 °C until further use. Normal-phase HPLC connected with a fluorescence detector was used to check the residual tocopherols according to the method described by Schwartz et al. (2008). The results showed no detectable residues of tocopherols.

Heptane portion of the purified oil-in-heptane solution was evaporated under nitrogen flow followed by addition of protein extracts in water and a brief coarse emulsion-making procedure using Ultra-Turrax®. Oil-in-water emulsions were prepared with a final concentration of 5% (w/v) oil and 1% (w/v) quinoa and amaranth proteins in study II while the composition was 10% (w/v) oil and 3% faba bean proteins and soy protein isolate in study III. Final stable emulsions were obtained by homogenizing the coarse emulsions

via a high-pressure M-110Y Microfluidizer® (Microfluidics™, MFIC Corp., MA, USA) at an operating pressure of 600 bars for 10 min of continuous flow process. Next, sodium azide was added into the emulsions with a final concentration of 0.02% (w/v) to prevent possible microbial growth. Oil-in-water emulsions stabilized with Tween®20 instead of proteins were prepared with the same method as control group (II).

4.5 OXIDATION MODELS (I-III)

Experimental setup conditions of oxidation models are summarized in Table 3 while overall schematic representation of the experimental work is summarized in Figures 8 (study I), 9 (study II), and 10 (study III). Samples to be incubated were placed in vials with screw caps and thus oxygen was available in the headspace of these vials.

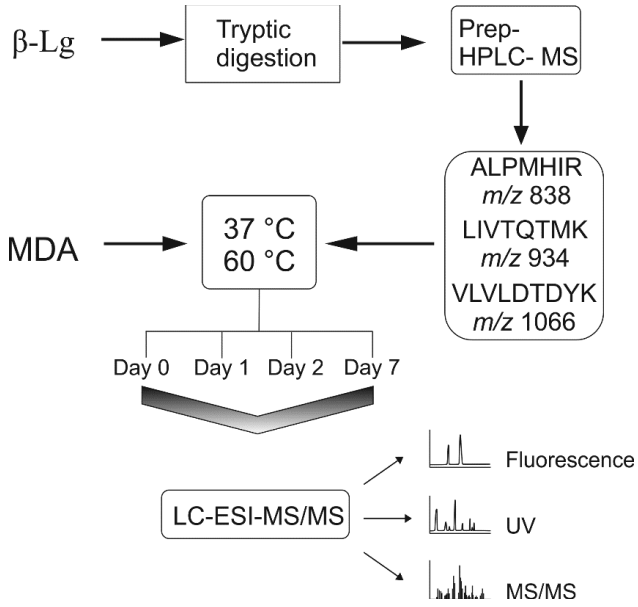

Figure 8 Representative scheme of experimental work in study I.

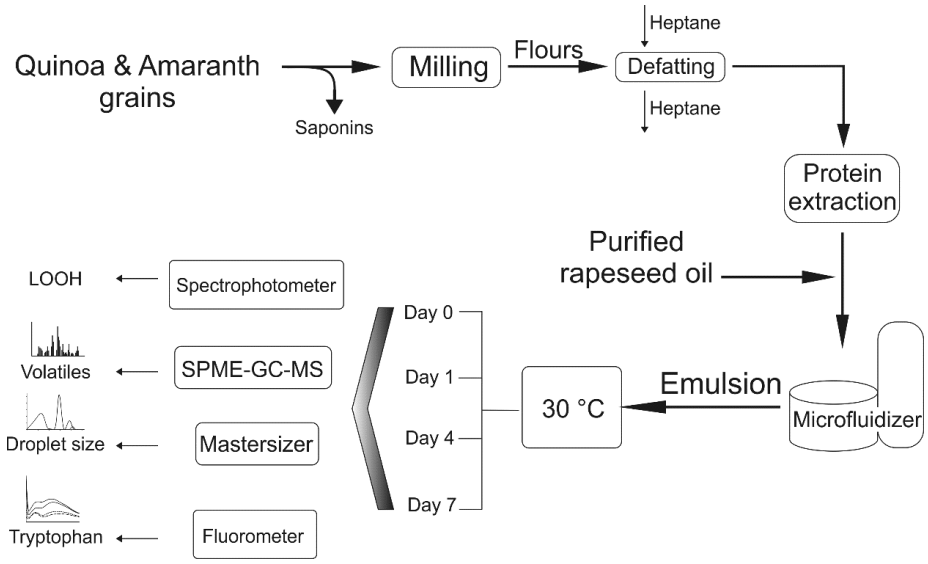

Figure 9 Representative scheme of experimental work in study II.

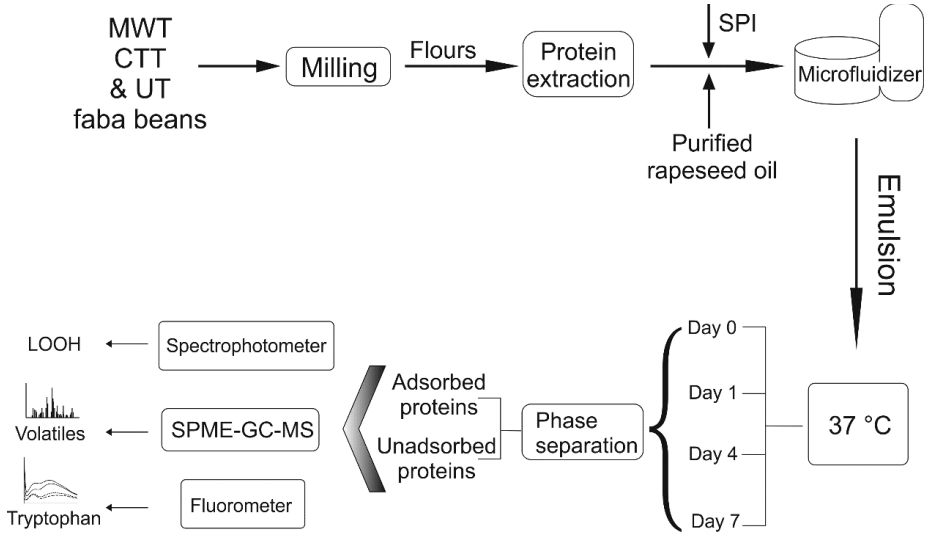

Figure 10 Representative scheme of experimental work in study III.

Malondialdehyde (MDA) used in study I was prepared through acid hydrolysis of TMP according to Adams et al. (2008) and Wu et al. (2009b). TMP hydrolyzed with 0.1 N HCl and kept at 37 °C for an hour in the dark, in order to obtain 100 mM of MDA. Concentration of MDA was verified with a

spectrophotometer at 245 nm (ε = 13700). The pH of MDA solution was adjusted to 7.3-7.4 with 6 M NaOH. Selected β-Lg peptides were dissolved in 10 mM ammonium bicarbonate buffer (pH adjusted to 7.3-7.4 with acetic acid) and mixed with MDA solution in Eppendorf tubes® with a final concentration of 5 mM MDA. Incubation samples with MDA and control samples containing only the peptides were prepared in triplicates and placed at 37 °C and 60 °C. Sample aliquots were taken on days 0, 1, 2, 7 and kept at −70 °C until LC-MS analyses.

Emulsion samples were stored at 30 °C (II) and 37 °C (III) in the dark with a gentle stirring action by a magnet in order to maintain similar conditions in all emulsion sample groups. Analytical samples were collected from emulsions to monitor the oxidative changes and emulsion stability on days 0, 1, 4, and 7. Analyses were performed in triplicates.

Table 3 Conditions of oxidation models used in the studies.

Study	Protein / Lipid	Concentration	Storage temperature	Sampling days
I	β-Lactoglobulin peptides	~10 mg/mL	37 and 60 °C	0, 1, 2, and 7
	Malondialdehyde	5 mM		
II	Quinoa and Amaranth proteins	1% (w/v)	30 °C	0, 1, 4, and 7
	Stripped rapeseed oil	5% (w/v)		
III	Faba bean proteins	3% (w/v)	37 °C	0, 1, 4, and 7
	Soy protein isolate	3% (w/v)		
	Stripped rapeseed oil	10% (w/v)		

4.6 OXIDATION ANALYSES

4.6.1 LC-MS ANALYSES OF MDA INTERACTIONS WITH β-Lg PEPTIDES (I)

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) technique was employed to follow MDA-induced modifications of selected β-Lg peptides. HPLC equipment was coupled with a Bruker Esquire (Bremen, Germany) quadrupole ion trap mass spectrometer (QIT-MS) through an electrospray ionization interface (ESI). The reversed phase column for all the analyses was Waters XBridge BEH130 C18 (particle size 3.5 µm, 2.1 x 100 mm) with which a guard column was used. Throughout the LC-MS runs, injection volume was 10 µL, column temperature was set at 30 °C and flow rate was kept constant at 0.35 mL/min. Mobile phase eluents were 0.1% formic acid (v/v) in water and 0.1% formic acid (v/v) in acetonitrile. UV detection was carried out at 205 nm by diode array detector

along with 280 nm (for amino acids with aromatic side-chains). Fluorescence detector was set at 395/ 460 nm (ex/ em).

Throughout the analyses in-house reference peptide (*m/z* 1246) runs were added to each peptide sample set at certain injection intervals. The reference peptide was isolated in ion trap to examine the day-to-day variances of the mass analyzer. The MS parameters used for the ionization source and the mass analyzer were set as described by Koivumäki et al. (2012). Theoretical singly-charged monoisotopic fragment ion patterns of peptides that result from MS/MS analysis were obtained from the Institute for System Biology website by addition of expected MDA adducts on amino acid residues and peptide N-termini using the Proteomics Toolkit MS/MS Fragment ion calculator (http://db.systemsbiology.net/proteomicsToolkit/).

4.6.2 ANALYZING OXIDATION AND PHYSICAL STABILITY IN EMULSIONS PREPARED WITH PROTEINS (II-III)

Lipoxygenase (LOX) activity measurement in faba bean flours (III) was based on methods described by Axelrod et al. (1981), Gökmen et al. (2002) and Jiang et al. (2016). Enzyme was water-extracted from 0.5 g of flour samples followed by centrifugation for 10 min at 15200 *g*. Supernatant was collected and filtered through to be used as the enzyme extract. Equal amounts of linoleic acid and Tween®20 were mixed with water and 300 µL of 1 N NaOH was added to clarify the solution. Afterwards, 0.2 mL of linoleate substrate (10 mM) was added to 2.6 mL 0.1 M sodium phosphate buffer (pH 6.0) and mixture was placed in a water bath of 25 °C. Subsequently, 0.2 mL of enzyme extract was added into the mixture to start the enzymatic reaction and stored for 5 min before 3 mL of 0.1 N NaOH was added to the mixture to stop the enzyme activity. For control samples, the same procedure was followed in which 0.2 mL water was added to substrate mixture instead of the enzyme extract. UV absorbance of conjugated dienes (CD) in samples was measured at 234 nm using a UV/Vis spectrophotometer (Lambda 25, PerkinElmer Inc., MA, USA). LOX activity in samples was expressed as µmol CD per g sample per minute with molar absorptivity value of 25000 cm^{-1} M^{-1}. The same method was applied also for quinoa and amaranth flours in Study II, the results of which showed no considerable endogenous enzyme activity.

Progress of lipid oxidation in the emulsions was monitored via CD formation and secondary oxidation volatile compounds. CD formation was measured according to a modified method by Lethuaut et al. (2002). An aliquot of the emulsion sample was mixed with 2-propanol and shaken to extract CD into the solvent phase which was followed by centrifugation at 14000 *g* for 20 min. The resulting supernatant was collected and measured at 234 nm using a UV/Vis spectrophotometer (Lambda 25 UV/Vis

Spectrophotometer, PerkinElmer, USA). Concentration of CD was expressed as mmol CD/ kg oil (Molar absorptivity, $\varepsilon = 25000\ M^{-1}\ cm^{-1}$).

Formation of volatile lipid oxidation products was monitored by detection and identification via headspace-SPME-GC-MS technique. Integrated peak areas of detected and selected volatile compounds were used to compare the progress of oxidation within an emulsion group between sampling days rather than quantification of these volatiles. Emulsion aliquots were collected on sampling days in headspace-SPME-GC vials closed with screw caps. The equipment consisted of an SPME injector, a GC, and a MS detector. The volatiles were extracted using a divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber assembly with 50/30 μm film thickness. Chromatographic separation of compounds was achieved through a SPB®-624 capillary column with dimensions of 30 m x 0.25 mm i.d. and 1.4 μm film thickness (Supelco, PA, USA). The SPME-GC-MS method employed in the study was developed by Damerau et al. (2014). Equilibration step was carried out at 40 °C followed by extraction at 40 °C. Next, fiber was desorbed at 250 °C at the GC front inlet in "spitless" mode. The details for the GC operation can be found in the original papers (II-III). Ionization energy for MS detection was 70 eV and *m/z* scan range was 40-300 Da. Identification of the volatile compounds was based on mass spectral data library Wiley 7N (Wiley Registery™ of Mass Spectral Data, 7th ed., USA) and retention times of these compounds in previously published data (Damerau et al. 2014).

Progress of protein oxidation in emulsions within sampling days was assessed through the changes in tryptophan fluorescence. In study II emulsion aliquots were dissolved in phosphate buffer (pH 7.4). In study III modifications to proteins were examined via changes in tryptophan fluorescence of proteins that are located both in continuous phase (unadsorbed proteins) and lipid interface (adsorbed proteins) based on a method reported by Chapleau and de Lamballerie-Anton (2003) and Mikkonen et al. (2016). Sample aliquots collected in Eppendorf® tubes were centrifuged at 10500 *g* for 20 min to separate phases in the emulsion. Subsequently, part of the aqueous fraction was taken for fluorescence measurements while an aliquot of the cream layer was collected to a different container. In order to separate the adsorbed proteins from the lipid interface, 1% SDS solution was added to the collected cream portion and centrifuged at 16000 *g* for 15 min. As a result of the newly separated phases, aqueous portion contained the adsorbed proteins of initial emulsion. An aliquot of this phase was collected for fluorescence spectroscopy. Following necessary dilutions fluorescence samples were measured in quartz cuvettes using a fluorometer (LS 55 Luminescence Spectrometer, PerkinElmer Inc., MA, USA) (II-III). Emission spectra were collected between 300 and 400 nm upon excitation at 283 nm (Estévez et al. 2008).

For study II changes in droplet size during the course of experiments were monitored via a laser diffraction particle size analyzer (Mastersizer Hydro 3000 SM, Malvern Instruments Ltd., Worcestershire, UK). The parameters entered into the Mastersizer software included the refractive index for rapeseed oil as 1.47 and for water as 1.33 while oil density was set as 0.905 g/cm^3. Another set of emulsion samples of all groups were also stored at 6 °C in order to detect differences in emulsion stability. Analytical samples were diluted in water during measurement and data on droplet size distribution, volume mean diameter: D[4,3]; surface mean diameter: D[3,2]; and median droplet size: D_v50 were collected. Specific surface area (SSA) of droplets was calculated according to the equation (Lethuaut et al. 2002):

$$SSA = \frac{6\,\emptyset}{D[3,2]}$$

where Ø is the volumetric oil fraction and D[3,2] the surface mean diameter of the droplets. SSA is expressed in m^2/mL.

Statistical analyses of data were carried out using SPSS software (IBM Corp., v.24.0.0.1). Tukey's HSD test was employed as a post-hoc analysis with ANOVA to mark the significant mean differences at a level of 0.05 (n=3).

5 RESULTS

5.1 REACTIONS OF MDA AND β-Lg PEPTIDES (I)

Isolated and selected tryptic peptides of β-Lg peptides were detected with $[M+H]^{1+}$ *m/z* values of 838 (Ala-Leu-Phe-Met-His-Ile-Arg, abbreviated as ALPMHIR), 934 (Leu-Ile-Val-Thr-Gln-Thr-Met-Lys, abbreviated as LIVTQTMK), and 1066 (Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys, abbreviated as VLVLDTDYK). Incubation of these peptides with MDA led to formation of two distinct adducts. These compounds were detected via LC-ESI-MS/MS analyses with *m/z* value increments of +54 and +134 Da compared to the original peptides. Moreover, a distinct intensity of fluorescent peak was detected at the same retention time of modified peptides with increased *m/z* values of +134 Da.

Day 0 samples of ALPMHIR control group and those incubated with MDA contained two oxidation products of ALPMHIR observed as single oxygen molecule addition to the peptide (+16 Da, *m/z* 854). These oxidized peptides, most likely generated during sample preparation, displayed no major changes in abundance in control samples during the storage period due to lack of a promoted oxidizing system in the incubation medium. However, their peak area signal decreased considerably in samples containing MDA and were not detected after day 2 in samples at 60 °C. Unmodified peptide ALPMHIR (*m/z* 838) was detected with retention time (RT) of around 22.30 min in all samples. Peak area signals of *m/z* 838 in control samples decreased only in minor levels, while in samples with MDA this peptide displayed a considerable amount of reduction in peak area, especially at 60 °C (Figure 11). On the other hand two distinct MDA-modified peptides were detected with *m/z* values of 892 (ALPMHIR + 54 Da) at RT ~33.60 min and 972 (ALPMHIR + 134 Da) at RT 38.50 min. Both modified peptides were found to form faster at 60 °C compared to samples at 37 °C, however the levels of modified peptide with *m/z* 892 displayed a downward trend after day 1 at 60 °C (Figure 11). Tandem mass spectrometry was applied to characterize the modified peptides *m/z* 892 and 972. Comparison of observed MS/MS fragment ions with the fragment ions resulting from theoretical modifications on side-chains and N-terminus amino acid residues of ALPMHIR revealed the modified sites of the peptide. These were characterized as adducts on His side-chain for *m/z* 892 and the N-terminus of Ala residues for *m/z* 972.

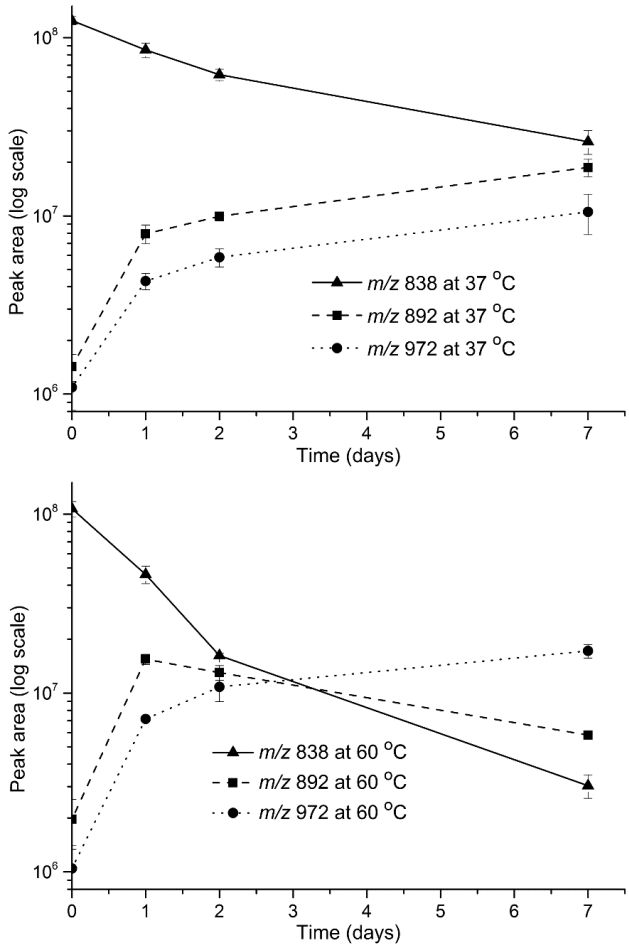

Figure 11 Changes in abundance of peptide ALPMHIR (*m/z* 838) and its MDA adducts *m/z* 892 (Δ54) and 972 (Δ134) during storage at 37 °C (upper) and 60 °C (lower).

In samples containing LIVTQTMK (*m/z* 934) the unmodified peptide had a retention time of around 26.00 min. As in previous peptide, oxidized form of LIVTQTMK was also observed with +16 Da (*m/z* 950) on day 0 of both control and MDA-containing samples. This peptide was detected at RT ~19.00 min. While the detected levels of *m/z* 950 slightly increased in control samples, those incubated with MDA displayed a decreasing trend in levels of this oxidized peptide, especially at 60 °C. The unmodified peptide also exhibited a decrease in peak signal during incubation with MDA in a faster manner at 60 °C than at 37 °C (Figure 13). MDA-modified peptides were detected with *m/z* values of 988 (LIVTQTMK + 54 Da) and 1068 (LIVTQTMK + 134 Da), the latter one displaying fluorescent properties. Both of these modified peptides demonstrated an increasing trend of formation at

37 °C. Meanwhile at 60 °C, after rapid formation within 2 days, *m/z* 988 had a lower peak signal on day 7. Similarly, formation of *m/z* 1068 after day 2 slowed down (Figure 12). On the other hand oxidized peptide *m/z* 950 that was detected on day 0 was also found to have undergone MDA-triggered modifications during storage. Although in minute levels, two modified peptides with *m/z* values of 1004 (LIVTQTMK + 16 + 54 Da) and 1084 (LIVTQTMK + 16 + 143) were also detected. MS/MS analyses on MDA-modified peptides *m/z* 988 and 1068 showed that the peptide adduct sites were Gln side-chains for both compounds.

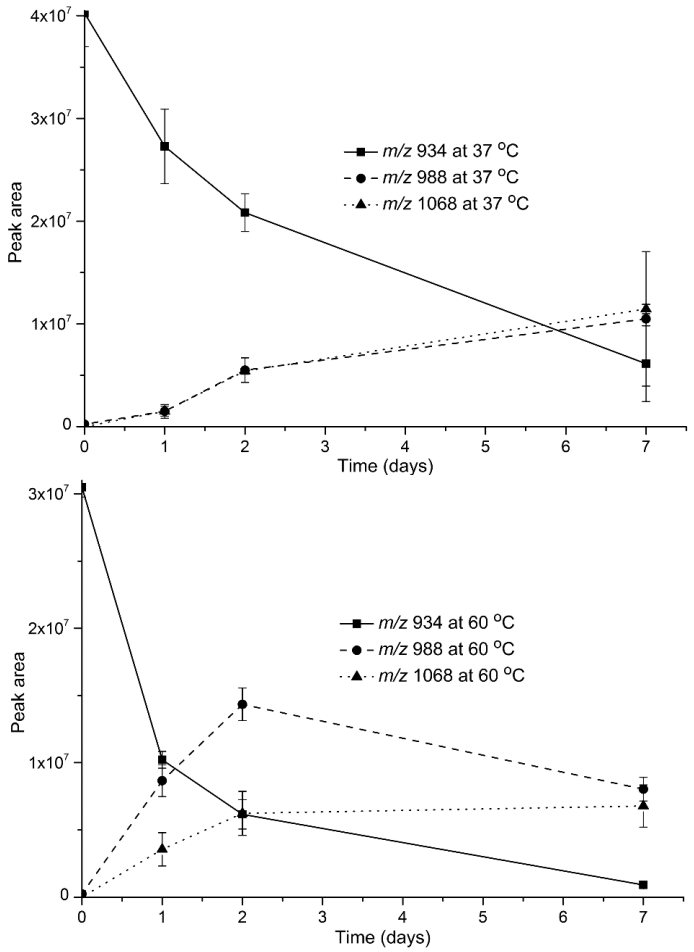

Figure 12 Changes in abundance of peptide LIVTQTMK (*m/z* 934) and its MDA adducts *m/z* 988 (Δ54) and 1068 (Δ134) during storage at 37 °C (upper) and 60 °C (lower).

Samples with VLVLDTDYK (*m/z* 1066) were found to contain cross-linked peptides via formation of dityrosine which were retained later than the

unmodified peptide with *m/z* value of 1066 as doubly charged molecular ion $[M+2H]^{2+}$. However, this dipeptide was found in much lower levels than the unmodified peptide.

Unmodified peptide VLVLDTDYK was retained at min 28.70 and the peak signal intensity displayed no major changes in control samples. On the other hand, detection levels of the main peptide were much lower at the end of storage period in samples incubated with MDA as expected. Peak signals of this peptide decreased in a considerable amount at 60 °C compared to 37 °C (Figure 15). MDA-triggered modifications on VLVLDTDYK were observed in newly formed compounds with *m/z* values of 1120 (VLVLDTDYK + 54 Da) and 1200 (VLVLDTDYK + 134 Da). Formation of *m/z* 1120 was in a more rapid fashion at 60 °C than at 37 °C. However the levels of this modified peptide demonstrated a slow reduction after day 2 in samples at 60 °C, while accumulation of *m/z* 1120 continued in samples stored at 37 °C (Figure 13). On the other hand *m/z* 1200 which exhibited strong fluorescent signals displayed a continuous increase in peak signals at both temperatures with samples at 60 °C showing a swifter formation than at 37 °C. MS/MS fragment ion patterns revealed that the two MDA-modified peptides with mass increments of +54 and +134 Da were adducts located at the N-terminus amino group of Val residues of the peptide VLVLDTDYK (Figure 14).

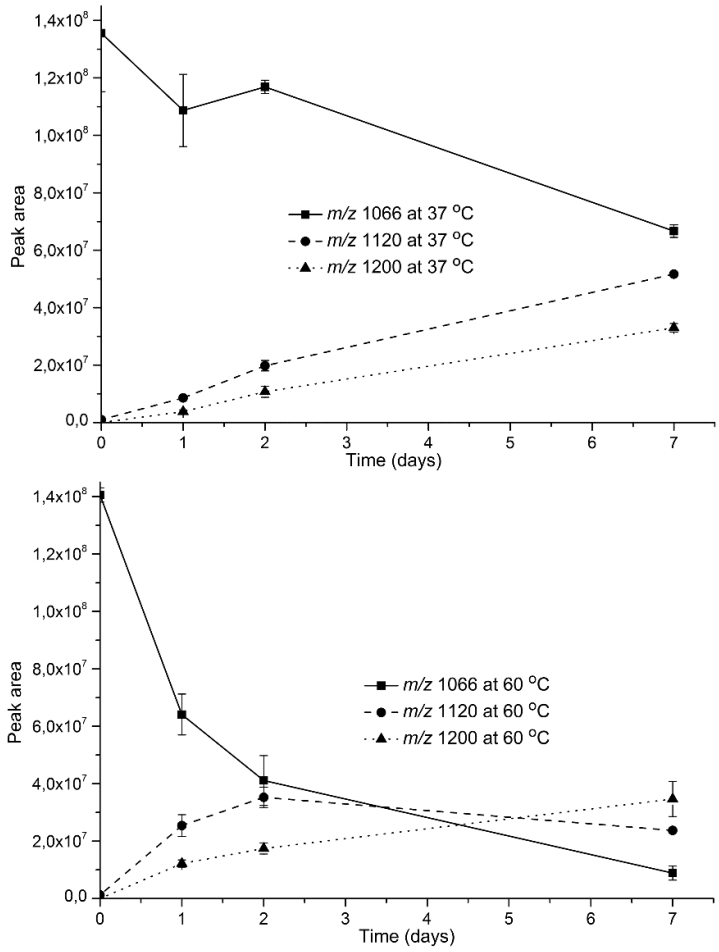

Figure 13 Changes in abundance of peptide VLVLDTDYK (*m/z* 1066) and its MDA adducts *m/z* 1120 (Δ54) and 1200 (Δ134) during storage at 37 °C (upper) and 60 °C (lower).

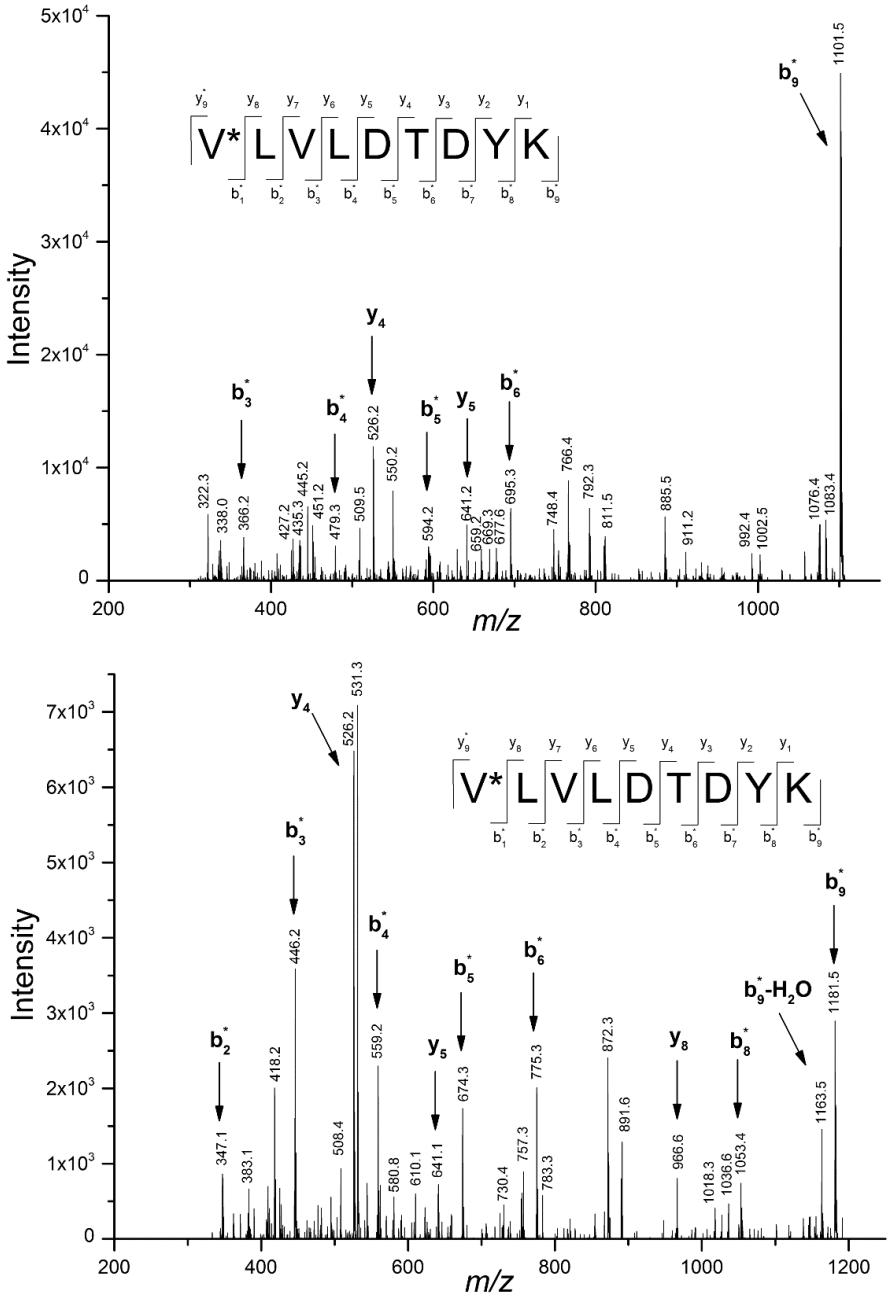

Figure 14 MS/MS spectra and fragment ions of MDA adducts of peptide VLVLDTDYK with *m/z* value of 1120 (upper) and 1200 (lower). Modified amino acid site on the peptide is denoted with asterisk (*).

5.2 OXIDATION OF EMULSIONS PREPARED WITH QUINOA AND AMARANTH PROTEINS (II)

Total protein content of quinoa and amaranth flours was measured as 13.02 (±0.12) and 15.93 (±0.51) g/100 g flour (dry weight), respectively. Moisture content of these samples were found as 11.8% (± 0.4) in quinoa flour and 11.3% (± 0.5) in amaranth flour (Ramos Diaz et al. 2015). Furthermore, protein content of water extracts was measured as 5.01 (±0.30) and 5.20 (±0.45) g/100 g flour (dry weight) in quinoa and amaranth flours, respectively.

Analyses of emulsion droplet size data obtained via Mastersizer provided with information on the volume mean diameter, $D[4,3]$; median diameter, D_v50, and surface mean diameter, $D[3,2]$ which is used to calculate specific surface area (SSA) of droplets in the emulsions. Mean SSA values for the freshly prepared emulsions were calculated as 3.69, 2.80, and 6.63 m^2/mL. In protein-stabilized emulsions SSA values decreased noticeably during storage at 30 °C. Table 4 presents the values for physical parameters examined with the particle size analyzer during storage of the emulsions at 30 °C.

Table 4 Emulsion droplet size parameters during storage at 30 °C.

Emulsions	Oxidation days	Volume mean diameter, D[4,3] (μm)	Median diameter, Dv 50 (μm)	Specific Surface Area, SSA (m^2/ mL)
Quinoa	day 0	7.81 ± 1.89	0.15 ± 0.00	3.69 ± 0.02
	day 1	24.30 ± 6.91	10.28 ± 0.74	0.03 ± 0.00
	day 4	69.37 ± 1.42	33.63 ± 0.45	0.01 ± 0.00
	day 7	45.90 ± 0.85	35.00 ± 0.20	0.01 ± 0.00
Amaranth	day 0	15.27 ± 5.86	0.20 ± 0.01	2.80 ± 0.06
	day 1	8.61 ± 0.03	8.44 ± 0.03	0.04 ± 0.00
	day 4	50.80 ± 3.97	46.83 ± 3.47	0.01 ± 0.00
	day 7	85.53 ± 17.62	34.43 ± 2.34	0.48 ± 0.01
Tween®20	day 0	0.64 ± 0.22	0.06 ± 0.00	6.63 ± 0.23
	day 1	0.37 ± 0.18	0.07 ± 0.00	6.39 ± 0.01
	day 4	0.75 ± 0.16	0.07 ± 0.00	6.37 ± 0.02
	day 7	0.09 ± 0.00	0.07 ± 0.00	6.44 ± 0.00

* Values denote mean ± standard deviation (n = 3)

Droplet size distributions showed that majority of the droplets in newly prepared emulsions produced with high-pressure homogenizer was under 1 μm (Figure 15). However, the distribution of droplet size changed considerably during storage in quinoa- and amaranth-stabilized emulsions at 30 °C (Figure 15a–b). No major variations were observed in Tween®20-

stabilized emulsions (Figure 15c). The extent of modifications detected in droplet size distributions was minor in protein-stabilized emulsions stored at 6 °C (Figure 15d–f). At both temperatures emulsions prepared with amaranth proteins maintained better physical stability than those with quinoa proteins.

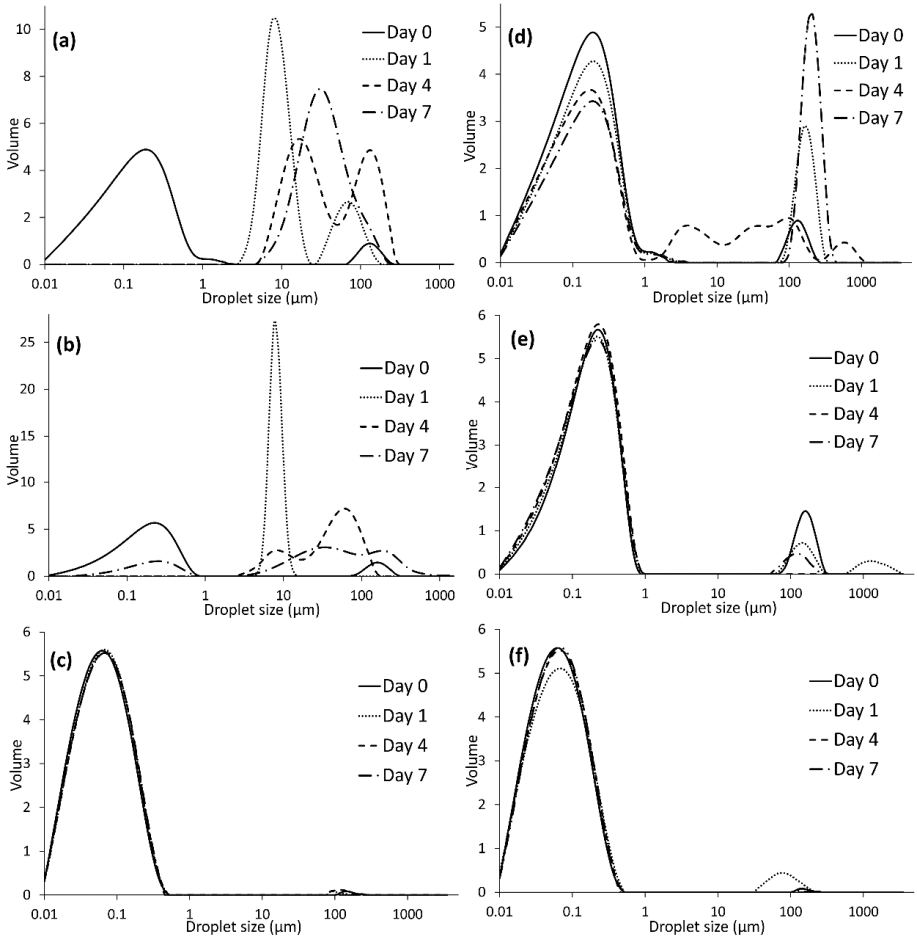

Figure 15 Changes in droplet size distributions of emulsions during storage. (a) quinoa protein-emulsions at 30 °C; (b) amaranth protein-emulsions at 30 °C; (c) Tween®20 emulsions at 30 °C; (d) quinoa protein-emulsions at 6 °C; (e) amaranth protein-emulsions at 6 °C; (f) Tween®20 emulsions at 6 °C.

Formation of CD in all emulsions stored at 30 °C followed a similar pattern until day 4, after which protein-stabilized emulsions displayed an increased yield of CD, while Tween®20-stabilized-emulsions demonstrated a slower rate CD formation (Figure 16).

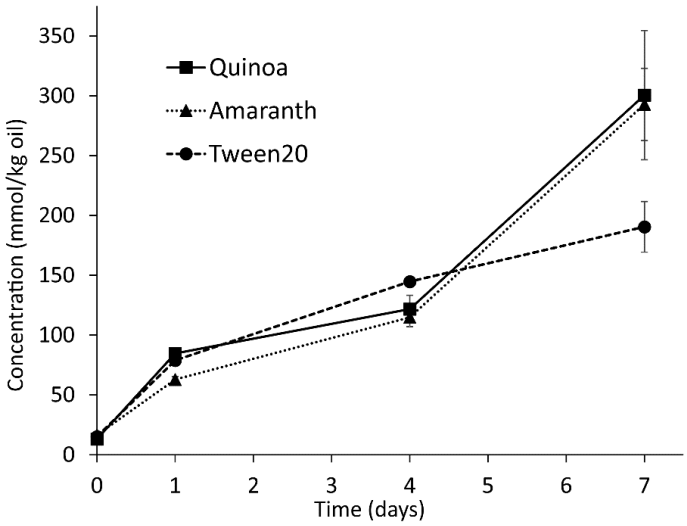

Figure 16 Conjugated diene hydroperoxide (CD) formation in emulsions during storage at 30 °C.

Among secondary oxidation compounds detected with SPME-GC-MS, ten most prominent ones were selected and monitored for the progress of oxidation in emulsions. These compounds included 2-pentylfuran, hexanal, 2-octenal, 2-heptenal, 2,4-heptadienal, 2-pentenal, 3,5-octadien-2-one, 2,4-hexadienal, 5-pentyl-2(5H)-furanone, and nonanal. Figure 17 presents the formation of volatiles in emulsions during storage. Formation of secondary oxidation products was noticeably more advanced in quinoa protein-stabilized emulsions compared to amaranth protein-stabilized emulsions. Occurrence of 2,4-hexadienal, 5-pentyl-2(5H)-furanone, and nonanal was more particular to protein-stabilized emulsions than Tween®20 emulsions. Meanwhile, 2-heptenal and 2-pentenal had a more pronounced rate of formation in Tween®20 emulsions. Accumulation of 2-pentylfuran, hexanal, 3,5-octadien-2-one, 2,4-hexadienal, and nonanal was more pronounced in quinoa protein-emulsions compared to other emulsions on day 7. In all emulsions 2-pentylfuran, 2-octenal, and 5-pentyl-2(5H)-furanone formations were emphasized starting from day 4 measurements while nonanal displayed a presence already on day 0 and continued to increase in abundance in all emulsions.

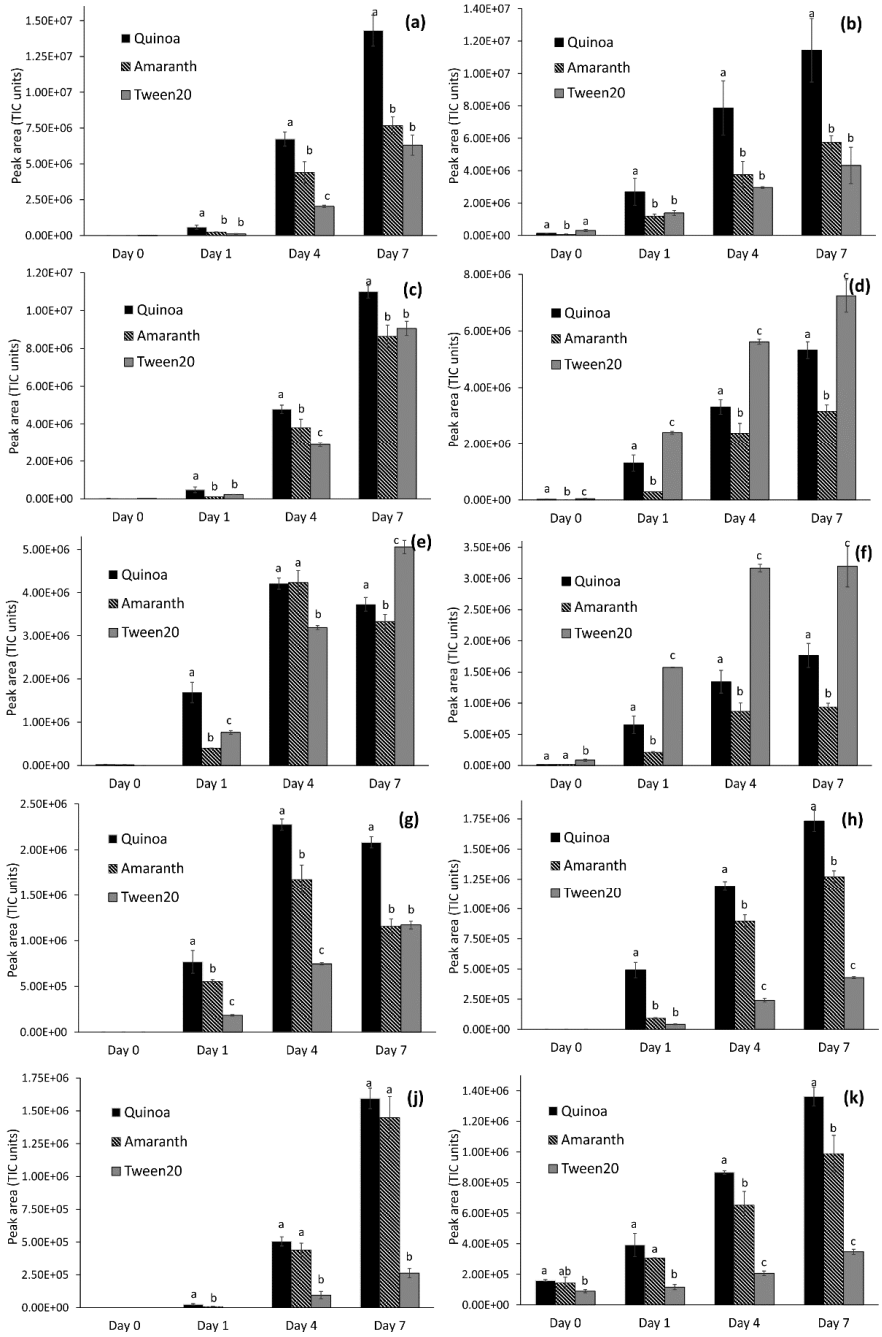

Figure 17 Volatile formation in emulsions with quinoa proteins (black), amaranth proteins (pattern fill), and Tween®20 (gray) at 30 °C. (a) 2-pentylfuran; (b) hexanal; (c) 2-octenal; (d) 2-heptenal; (e) 2,4-heptadienal; (f) 2-pentenal; (g) 3,5-octadien-2-one; (h) 2,4-hexadienal; (j) 5-pentyl-2(5H)-furanone; (k) nonanal. Significant mean differences between emulsions within analysis day are marked with different lowercase letters (p<0.05).

Protein oxidation led to loss of Trp fluorescence in both emulsions stabilized with quinoa and amaranth proteins. Fluorescence peak maxima displayed a so-called red shift from 345 to 353 nm in quinoa proteins and 344 to 358 nm in amaranth proteins. Oxidative degradation of proteins followed different patterns in these protein-stabilized emulsions (Figure 18). The largest loss of Trp fluorescence took place between days 0 and 1 in quinoa proteins, whereas in amaranth proteins the biggest reduction occurred between days 1 and 4. In quinoa protein-stabilized emulsions day 1 fluorescence intensity was measured as 54% of the initial day 0 fluorescence, while on day 4 the intensity was found to be ~38% of day 0. On day 7 no major change was observed compared to day 4. On the other hand, on day 1 amaranth protein fluorescence intensity was measured as 85.5% of day 0 intensity which declined to a level of 45.7% as measured on day 4. Finally, day 7 fluorescence emission intensity was found to be 34.9% of initial fluorescence.

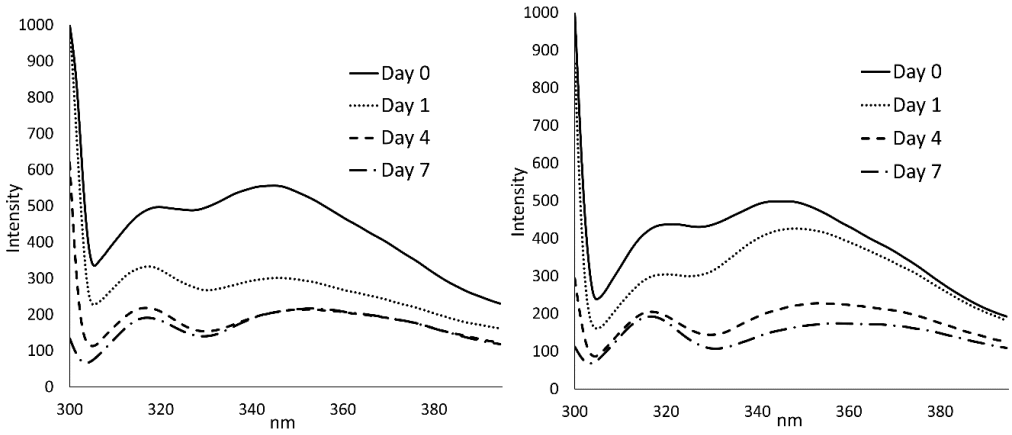

Figure 18 Spectra of tryptophan fluorescence during oxidation at 30 °C in quinoa (left) and amaranth (right) protein containing emulsions (ex. 283 nm).

5.3 OXIDATION OF EMULSIONS PREPARED WITH FABA BEAN PROTEINS (III)

Lipoxygenase (LOX) activity was found to be remarkably lower in both microwave-treated (MWT) and conventional thermal-treated (CTT) faba bean flours compared to the untreated (UT) faba beans. CTT samples displayed an inhibition of 96% in LOX activity while in MWT this level was at around 77% (Table 5).

Table 5 Lipoxygenase (LOX) activity measured in faba bean flours.

Sample name	Lipoxygenase activity (± SD*) (µmol CD** / g sample/ min)	% inhibition of native enzyme
Untreated faba bean flour	56.5 ± 0.9	
Microwave-treated faba bean flour	12.9 ± 1.9	77.3
Heat-treated faba bean flour	2.3 ± 0.1	96.0
Control sample	1.4 ± 0.6	

* SD: Standard deviation.

** CD: Conjugated diene hydroperoxides.

The highest amount of CD formation was measured in MWT and UT emulsions. While decomposition of CD was more emphasized in MWT emulsions after day 1, in UT emulsions CD decomposition rate was higher than the formation as found in measurements performed on day 7. Meanwhile CTT emulsions did not exhibit an abundant formation of CD throughout the storage period at 37 °C. Oxidation in emulsions prepared with soy protein isolate (SP) caused a steady increase in CD formation although it did not reach levels as advanced as in UT and MWT emulsions (Figure 19).

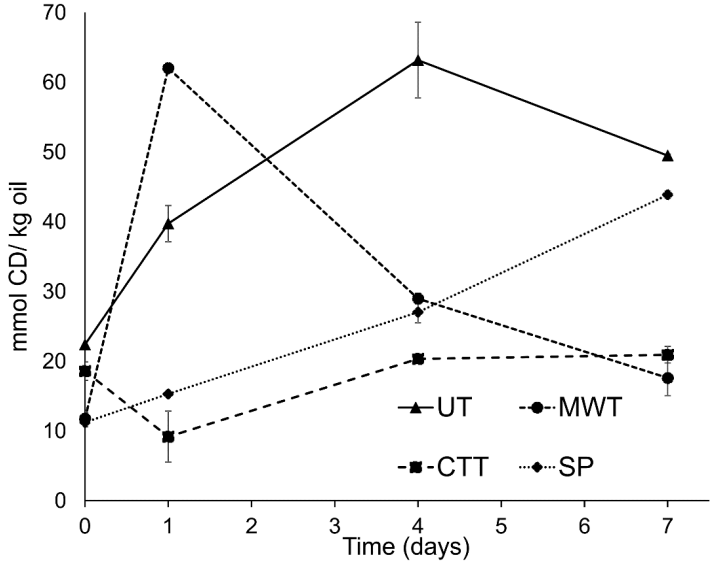

Figure 19 Formation of conjugated dienes (CD) during storage at 37 °C in emulsions stabilized with proteins from untreated (UT), microwave-treated (MWT), conventional thermal-treated (CTT) faba beans and soy protein isolate (SP).

Prominent volatiles detected and selected to be monitored as oxidation markers that are in common with all emulsions included aldehydes 2-pentenal, 2-hexenal, 2-heptenal, octanal, 2,4-heptadienal, 2-octenal, and nonanal; ketones (E,Z)-3,5-octadien-2-one, 2-heptanone, 3-octen-2-one, and (E,E)-3,5-octadien-2-one; and alkylfuran 2-pentylfuran. Figure 20 presents the formation of these volatiles in units of integrated peak areas over a storage period of seven days at 37 °C. In general, the highest diversity and abundance of secondary oxidation products were observed in UT emulsions. In particular hexanal and 2-octenal were the two most dominant volatiles among others that were found in UT emulsions on day 7. In addition to the selected volatile compounds, UT emulsions also contained advanced oxidation products such as alcohols 1-pentanol, 1-octen-3-ol, and 1-heptanol, acids hexanoic acid and octanoic acid, and longer chain aldehydes such as 2,4-nonadienal and 2,4-decadienal. At the end of day 7, the most accumulated volatile in all emulsions was hexanal. Although lipid oxidation in UT emulsions progressed expansively, certain volatiles were more emphasized in other emulsions. These compounds were (E,Z)-3,5-octadien-2-one, 3-octen-2-one, and (E,E)-3,5-octadien-2-one. Apart from these ketones, formation of 2,4-heptadienal was dominant among other volatiles in emulsions of MWT, CTT, and SP proteins. Volatile formation rate and abundance were in a greater extent in MWT emulsions than in CTT emulsions. Compounds such as 2-pentenal, 2-hexenal, 2-heptenal, 2,4-heptadienal, 3-octen-2-one, 2-octenal, (E,Z)-3,5-octadien-2-one, and (E,E)-3,5-octadien-2-one accumulated in higher levels in MWT emulsions compared to CTT emulsions at the end of oxidation period. On the other hand, formation of 2-heptanone, 2-pentylfuran, octanal, and nonanal was more pronounced in CTT emulsions compared to MWT emulsions. Meanwhile, in SP emulsions some secondary oxidation products were detected in a noticeably higher levels on day 7 compared to emulsions stabilized by proteins from pre-treated faba beans. These volatiles included hexanal, 2-heptanone, 3-octen-2-one, (E,Z)-3,5-octadien-2-one, and (E,E)-3,5-octadien-2-one. Another marked observation in SP emulsions was the steep increase in formation rate of monitored volatiles during the last three days of oxidation period.

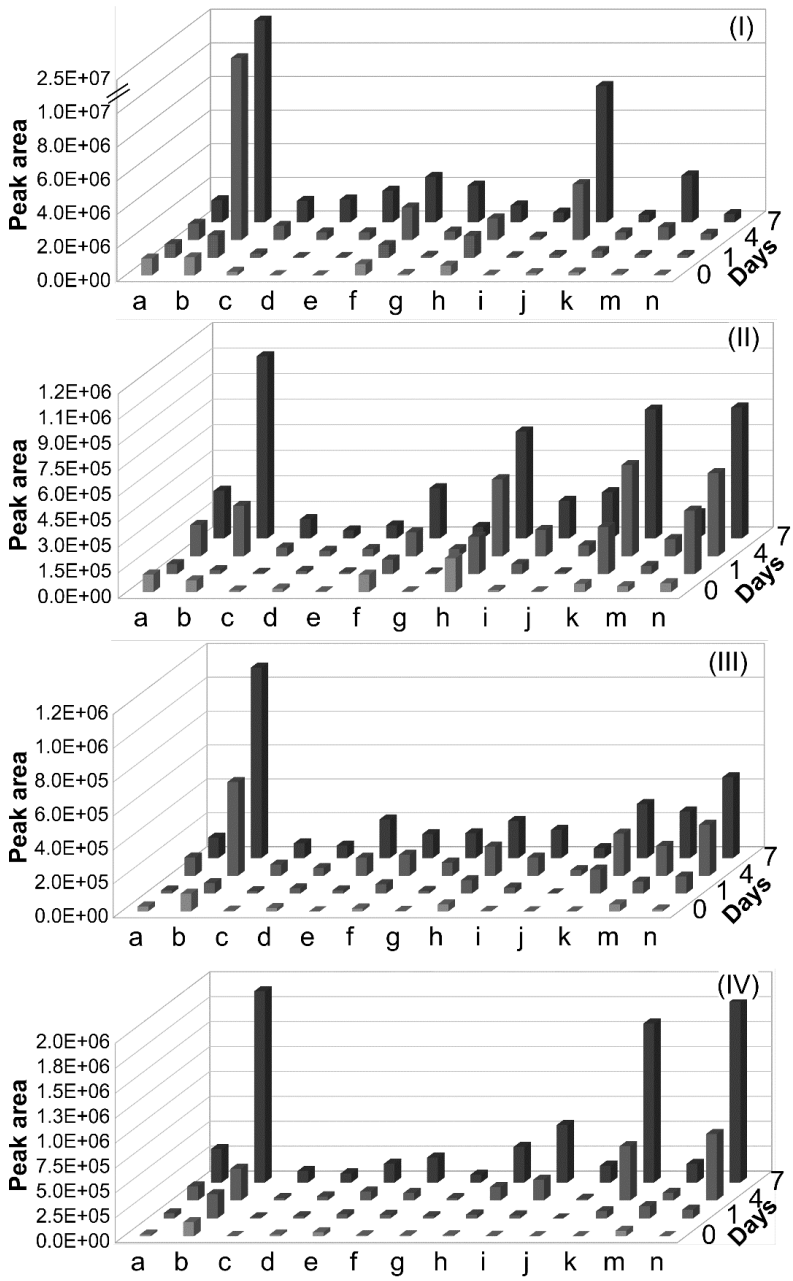

Figure 20 Formation of secondary oxidation volatiles observed in chromatographic peak areas during storage at 37 °C in (I) untreated; (II) microwave-treated; (III) conventional thermal-treated faba bean protein-, and (IV) soy protein-stabilized emulsions. Volatiles are denoted on x-axis as (a) 2-pentenal; (b) hexanal; (c) 2-hexenal; (d) 2-heptanone; (e) 2-pentylfuran; (f) 2-heptenal; (g) octanal; (h) 2,4-heptadienal; (i) 3-octen-2-one; (j) 2-octenal; (k) (E,Z)-3,5-octadien-2-one; (m) nonanal; (n) (E,E)-3,5-octadien-2-one.

Measurements of Trp fluorescence were carried out in proteins from cream phase (adsorbed proteins) and aqueous phase (unadsorbed proteins) to monitor protein oxidation. Peak maxima of fluorescence emission spectra were observed at around 345 nm for adsorbed proteins and around 332 nm for unadsorbed proteins.

Changes in Trp fluorescence were presented in Figure 21 as percentage intensity of the fluorescence measurements performed on day 0. Oxidative degradation of interfacial proteins was more advanced and rapid in MWT emulsions compared to CTT emulsions. Adsorbed proteins from MWT emulsions were oxidized extensively between days 1 and 4, after which the fluorescence emission stayed almost level. Likewise in CTT emulsions adsorbed proteins underwent oxidative degradation more extensively between days 1 and 4. The highest loss of Trp fluorescence from the interfacial layer was observed in SP emulsions where the intensity was reduced almost by half of day 0 value. The largest loss of intensity in SP emulsions took place between day 0 and day 1. Meanwhile in UT emulsions Trp degradation in adsorbed proteins did not occur as distinctly as in other emulsions.

Protein oxidation in aqueous phase proceeded differently than in cream phase. Degradation of Trp fluorescence in continuous phase was not as pronounced as in proteins from oil-water interface in MWT, CTT, and SP emulsions. However, extensive oxidation of proteins in aqueous phase of UT emulsions took place during the last three days of storage as a 35% decrease in intensity was measured on day 7. Meanwhile in MWT emulsions Trp fluorescence intensity fell by 26% after day 1 and in unadsorbed proteins from CTT emulsions fluorescence intensity was reduced by 14% at the end of the oxidation period. At day 7 it was observed that unadsorbed proteins of MWT, CTT, and SP emulsions produced almost the same level of Trp fluorescence.

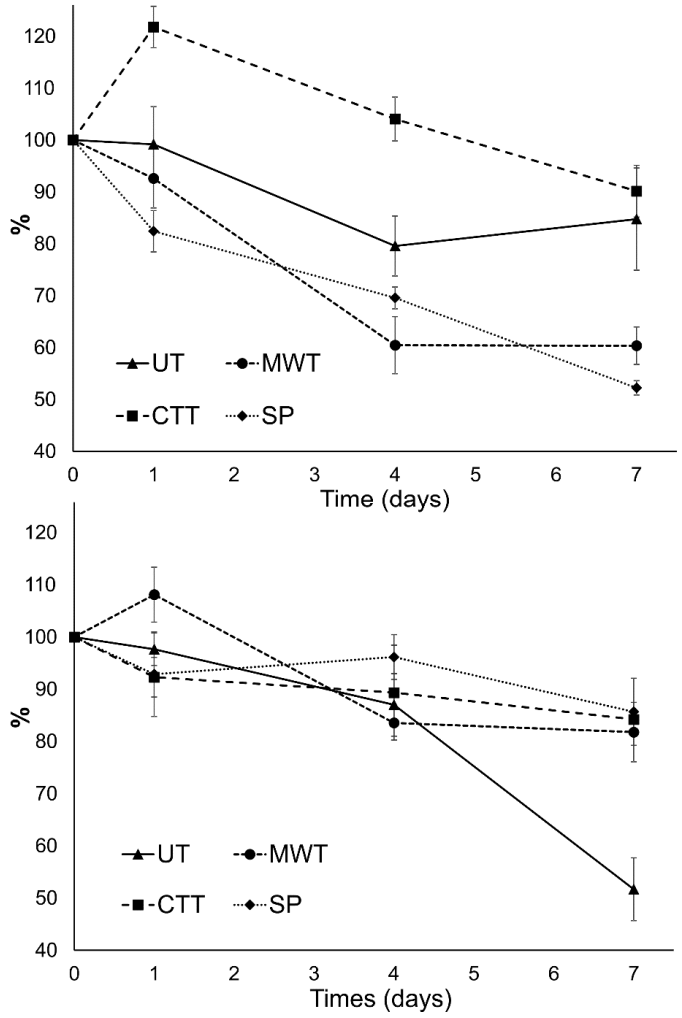

Figure 21 Changes in tryptophan fluorescence in adsorbed (upper) and unadsorbed (lower) proteins from emulsions stabilized with untreated (UT), microwave-treated (MWT), conventional thermal-treated (CTT) faba bean and soy proteins (SP).

6 DISCUSSION

6.1 INTERACTIONS OF MALONDIALDEHYDE WITH TRYPTIC β-LG PEPTIDES (I)

Selected β-Lg peptides ALPMHIR, LIVTQTMK, and VLVLDTDYK contained residues of Met, His, Tyr, Lys, Pro, Leu, Arg, and Gln which were reported to be prone to oxidative reactions and interactions with oxidized lipid species (Schaich 1980; Uchida 2003; Stadtman and Levine 2003; Davies 2005; Zhao et al. 2012). Accordingly, high susceptibility of these amino acid residues led to the occurrence of oxidized peptides during day 0 as detected with LC-MS. Oxidation may have taken place during preparation of incubation samples. However, no further oxidation was observed in either control samples or MDA-incubated samples.

The types of newly formed compounds with mass increments of +54 and +134 Da were common in all MDA-modified peptides, although the adduct sites varied. The depletion of the unmodified peptides was more severe at 60 °C than at 37 °C which shows that the MDA-triggered reactions were more advanced at the higher temperature as was reflected in the abundance of newly formed compounds at this temperature. Moreover, day 7 presence of the unmodified peptides in both temperatures and all incubation samples indicated that the amount of MDA molecules were the limiting factor in proceeding reactions. Therefore, the modified peptides detected were the preferred sites of reactions between MDA and the peptides.

6.1.1 DETECTION OF ENAMINAL-DERIVATIVE ADDUCT

An increase in *m/z* value of 54 Da indicates a Schiff base adduct formation between the amino group of the amino acid and MDA in a 3-amino-2-propenal-type (enaminal) structure (Uchida et al. 1997). This product was readily generated at both temperatures and a higher rate than the other adduct that was observed with the increment of +134 Da. The comparison between the formation rates at different temperatures showed that this Schiff base adduct was initially more prominent at the higher temperature, however after day 2, all peptide samples displayed a declining trend of this modified peptide at 60 °C. This observation was also reported by Slatter et al. (1998) as an attribution to labile nature of this adduct during prolonged periods of incubation. Thus, this compound is likely to break down and be involved in formation of other MDA-adducts such as the fluorescent dihydropyridine-type derivative (observed with +134 Da). The adduct sites of MDA displayed differences in each peptide as LC-MS/MS analyses indicated.

While His side-chain was preferred in ALPMHIR, covalent adduct was discovered to occur at Gln side-chain amino group of LIVTQTMK and at the N-terminal amino group of Val of peptide VLVLDTDYK. Among the amino acids that these peptides are composed of, MDA was expected to be reactive towards side-chains of His, Pro, Arg, Lys, and Gln residues. The observed sites of adducts indicate that under the conditions of this experimental work, the amino acids located at the C-terminal of the peptide were not as reactive towards MDA as the side-chain amino groups of Gln and His as well as the α-amino group of VLVLDTDYK. Moreover, in samples with peptide LIVTQTMK, the oxidized peptide observed at *m/z* 950 (+16 Da) was also found to have reacted with MDA to form Schiff base adducts. This modified peptide, even though in minor amounts, was detected with *m/z* value of 1004 (LIVTQTMK + 16 + 54) which showed that oxidized peptides are also prone to MDA-triggered modifications and thus adds to the complexity of oxidative interactions between proteins and lipids.

6.1.2 DETECTION OF DIHYDROPYRIDINE-TYPE ADDUCT

The other type of newly formed modified peptide in incubation samples observed with a +134 Da mass increase was characterized as the dihyropyridine (DHP)-type adduct. This structure is generated as a condensation product of Schiff-bases when the amino groups on amino acid side-chain or peptide N-terminus group react with more than one MDA molecule and stabilize in the form of a pyridine ring structure (Kikugawa et al. 1984). Kikugawa et al. (1981) reported this DHP-type product as one of the major fluorescent compounds that arise from reactions of MDA with amino acids. Thus, strong fluorescence properties of this Schiff base structure enable its identification with ease especially when combined with tandem mass spectrometry. In accordance, this DHP-type adduct was also confirmed with the increasing peak areas of chromatograms obtained during the incubation period via the fluorescence detection at 395/ 460 nm (ex/ em). While the accumulation of this adduct was favored at 60 °C, the formation rate was slower after day 2 in samples at the higher temperature compared to those stored at 37 °C. This indicates that at the higher temperature most of the MDA molecules initially react rapidly to yield the DHP-type adduct while as the concentration of MDA becomes limited for the Schiff base condensation reactions, the formation is retarded. The adduct sites for this type Schiff base formation included N-terminal amino group of Ala in ALPMHIR, side-chain amino group of Gln in LIVTQTMK, and N-terminal amino group of Val in VLVLDTDYK. As with the MDA-modified peptides with +54 Da, under the conditions of this experimental work, Arg and Lys residues were not the preferred sites of reactions with MDA and nucleophilic amino groups of N-terminal amino acids while Gln side-chains

were more reactive towards MDA. The common characteristics of both Arg and Lys side-chains under physiological pH utilized in this study (pH 7.4) were that they carry a positive charge below their side-chain pKa value which may have affected their reactivity towards MDA. In a similar fashion as was observed with the Schiff base adduct of +54 Da, oxidized form of peptide LIVTQTMK on the methionine side-chain (*m/z* 950) also was involved in formation of the DHP-type adduct detected at *m/z* 1084 (LIVQTMK + 16 + 134) in minor amounts. Formation of both types of Schiff base adducts with the oxidized LIVTQTMK explains the decrease in detection of the oxidized peptide peak signal.

Apart from the two prominent Schiff base adducts found with mass increments of +54 and +134 Da, no other adduct formations could be detected. Thus, these two types of MDA-triggered modifications of peptides were concluded to be reliable markers of interactions between MDA and the β-Lg peptides. Prolonged incubation periods at higher temperatures, however, may lead to decomposition of these adducts or further reactions such as cross-link formations. In this study LC-MS/MS method coupled with UV detection at 205 nm for peptide bonds and 280 nm for aromatic amino acid side-chains as well as monitoring the fluorescent compounds at 395/ 460 nm (ex/ em) proved to be an accurate technique to detect and characterize the marker compounds for MDA-triggered modifications of peptides. It was also evident from the results that oxidation of the peptides in terms of amino acid side-chain modifications was not as prominent as the interactions between MDA and β-Lg peptides.

6.2 OXIDATION OF PROTEINS AND LIPIDS IN EMULSIONS STABILIZED WITH PLANT PROTEINS (II-III)

6.2.1 FACTORS AFFECTING EMULSION STABILITY AND COURSE OF OXIDATION

In study II, saponins of quinoa and amaranth grains were washed away before the milling process in order to reduce foam formation and enable a more efficient emulsion-making. Chauhan et al. (1999) reported a reduction in emulsifying activity in the absence of saponins while they also found that this procedure increased the emulsion stability. The amount of water-soluble albumin and globulin fractions of quinoa and amaranth proteins may display variations between different cultivars. In this study, around 60% of total albumins and globulins from quinoa, and 75% of these proteins from amaranth were extracted based on the data from the review of literature

(Bressani and García-Vela 1990; Janssen et al. 2016). A higher yield of extraction would be possible with conditions of higher temperature and pH value (Guerreo-Ochoa et al. 2015), however these parameters were avoided not to cause unwanted oxidation and protein degradation during extraction process. Nevertheless, the repetition of protein extraction provided with enough amount of proteins to be utilized in the emulsion-making with the desired final protein concentration.

Monitoring the emulsion physical stability during storage with a particle size analyzer provided with information on the changes in the droplet size distributions and specific surface area (SSA) as oxidation advanced (study II). SSA is the total surface area of the interfacial layer around lipid droplets per unit volume. Thus, a smaller SSA value indicates the presence of larger droplets. Among the freshly prepared emulsions amaranth protein-stabilized emulsions yielded the lowest SSA value which means these emulsions had larger droplets compared to those prepared with quinoa protein. Accordingly, smaller droplets offer a larger surface area available at the beginning of the experiment for oxidative reactions at the interface of quinoa protein-stabilized lipid droplets. During the storage at 30 °C, droplet size distributions displayed noticeable changes in protein-stabilized emulsions while Tween®20-stabilized emulsions remained almost unchanged. It was reported that surfactant emulsifiers such as Tween®20 provide better stability against coalescence of lipid droplets than proteins (McClements and Decker 2000; Berton et al. 2012a). Due to their molecular rearrangement and inter-molecular interactions around droplets, surfactant emulsifiers form a more homogeneous and, accordingly, tighter interfacial layer compared to the heterogeneous and porous layer that proteins form due to their amphiphilic nature (Grigoriev and Miller 2009). Emulsions with quinoa proteins developed formation of larger droplets earlier than amaranth protein-emulsions which may be attributed to a more rapid rate of oxidative modifications. This was also supported with the degradation of tryptophan fluorescence as will be elucidated later. Droplet size analyses performed on the emulsions kept at 6 °C provided with different results than those at 30 °C. The presence of smaller droplets that were produced during the emulsion-making process evidently remained during the storage and in general protein-stabilized emulsions proved to be much more stable at 6 °C. The difference between two temperatures could be due to the greater extent of oxidation that would be expected at the higher temperature. Moreover, during cold storage quinoa protein-emulsions were clearly physically less stable than amaranth protein-stabilized emulsions as was the case with the higher temperature. Further studies may include a closer inspection of the relationship between other effects of temperature such as changes in continuous phase viscosity and protein oxidation.

In study III, activity of lipoxygenase enzyme (LOX) significantly influenced the course of oxidation in emulsions. Both microwave (MWT) and conventional thermal treatments (CTT) were found to reduce the enzyme activity in faba beans. LOX activity measurements in pre-treated faba bean flours showed that CTT was more effective in inhibiting the enzyme activity compared to MWT. Jiang et al. (2016) reported that both treatments were also adequate against the native peroxidase enzyme in faba beans. LOX uses *cis,cis*-1,4-pentadiene structures of polyunsaturated fatty acids as substrates which are present in fatty acids linoleic and linolenic acids of rapeseed oil. LOX catalyzes the direct formation of linoleic acid-derived hydroperoxides, namely 9-hydroperoxyoctadecadienoic and 13-hydroperoxyoctadecadienoic acids, without generating radicals (Schaich 2005). Furthermore, the activation energy required for the oxidation of linoleic acid is considerably lower than the autoxidation/ photooxidation pathway (Belitz et al. 2009) which would cause higher susceptibility of lipid species to enzymatic oxidation. Although the conditions for the highest activity for faba bean LOX was observed at 30 °C and pH 6.0, Al-Obaidy and Siddiqi (1981) reported that the enzyme remained active at temperatures up to 55 °C and a pH range of 4.0–8.0. Thus, a significant influence of LOX on the progress of oxidation was anticipated in emulsions containing the enzyme.

6.2.2 PROGRESSION OF LIPID OXIDATION

Early stages of lipid oxidation followed a similar pattern in both amaranth protein-, quinoa protein-, and Tween®20-stabilized emulsions (II). However after day 7, measurements showed that the net formation of CD was higher in protein-stabilized emulsions. This is most likely due to the propagation of hydroperoxide formation through interaction reactions driven by the radical species of proteins and lipids. Schaich (2005) described that the stages of oxidation are not isolated events but rather an ongoing series of reactions that give rise to radicals, oxidation products, and decomposition of these products to yield further radicals as well as stable end products. Therefore, it would be correct to assume that presence of reactive protein species are one of the main reasons that net CD accumulation is higher in protein-stabilized emulsions.

On the other hand, in study III, emulsions with CTT proteins exhibited the highest stability against formation of primary oxidation products, while MWT emulsions and UT emulsions contained the highest CD concentration. In UT emulsions increasing CD accumulation which was expected due to high LOX activity showed a downward trend after day 4 due to overwhelming hydroperoxide decomposition. Rapid CD formation in MWT took place within one day of storage which was followed by a steep fall in net CD formation. The initial extensive hydroperoxide formation can be attributed to

the combined effect of the remaining LOX activity post-treatment and radical-driven autoxidation propagated by protein radical species that were formed during microwave-treatment. On the other hand CD concentration in SP emulsions was found in increasing levels. During the last three days of oxidation highest rate of formation was detected in SP emulsions which was also the case for secondary oxidation products. This shows that propagation of the oxidation was more prolific and durable in SP emulsions while termination stage where stable end products arise was reached faster in faba bean protein-stabilized emulsions.

Analyses of secondary oxidation volatiles released in emulsions in study II showed that oxidation of quinoa protein-stabilized emulsions was the most advanced during seven days of storage at 30 °C. Major lipid oxidation products such as 2-pentylfuran, hexanal, 2-octenal, and nonanal were significantly more abundant in quinoa protein-emulsions. Compared to amaranth protein-emulsions, day 7 accumulation of monitored volatiles was more advanced in emulsions prepared with quinoa proteins. Moreover, day 1 measurements revealed earlier formation of these volatiles in emulsions with quinoa proteins. These results point to the higher rate of oxidation as well as the greater extent of oxidation in emulsions containing quinoa proteins compared to those with amaranth proteins. This finding was also supported by the earlier onset of droplet size increase as measured with particle size analyzer. One important factor for the higher oxidation rate may be the larger interfacial area of day 0 emulsions stabilized by quinoa proteins compared to amaranth protein-stabilized droplets. On the other hand, day 7 accumulation of three certain aldehydes, 2-heptenal, 2,4-heptadienal, and 2-pentenal was more prominent in Tween®20-stabilized emulsions compared to the protein-stabilized ones. The involvement of protein radical species throughout the propagation of lipid oxidation may have influenced the accumulation of certain volatiles while unsaturated aldehydes are known to be capable of undergoing further oxidation to generate new compounds that are not detected in emulsions with Tween®20 (Frankel 1982; Schaich et al. 2013). One such volatile whose formation was distinguished in protein-stabilized emulsions was 5-pentyl-2(5H)-furanone which could arise as an advanced oxidation product of hexanal, 2-pentylfuran, and octanal (Damerau et al. 2014).

In study III, UT emulsions experienced the most extensive and rapid lipid oxidation in terms of volatiles formed. Moreover, the detection of compounds that are associated with advanced lipid oxidation such as 1-pentanol, 1-octen-3-ol, 1-heptanol, hexanoic acid, and octanoic acid (Lampi et al. 2015) were particular to UT emulsions which indicated that oxidation progressed furthest in these emulsions. These volatiles were not observed in emulsions monitored in study II which supports the profound influence of enzymatic oxidation pathway. In addition to abundance of secondary linoleic

and linolenic oxidation products, formation of oleic acid products nonanal and octanal (Choe and Min 2006) was also observed in UT emulsions, however with a steep increased rate during the last days of storage. It was anticipated that these volatiles derived from oleic acid which is not oxidized via LOX pathway would arise at a later stage in oxidation. This is due to large accumulation of hydroperoxides that decompose and give rise to radicals involved in oleic acid oxidation. Thus, when compared with emulsions prepared with proteins from pre-treated faba beans, the heavy oxidative impact of LOX pathway is evident in UT emulsions. Furthermore, this oxidative effect of the remaining LOX activity post-treatment in MWT emulsions was also observed in formation of selected volatiles. A range of volatiles were found in higher abundance in MWT emulsions than in CTT emulsions. Among these, 2-pentenal and 2,4-heptadienal are oxidation products of linolenic acid while 2-octenal, 2-heptenal, and 3-octen-2-one are linoleic acid-derived volatiles (Frankel 1982; Damerau et al. 2014). Formation of 2-pentylfuran, a linoleic acid volatile, was more emphasized in CTT emulsions than in MWT emulsions, while another major linoleic acid product hexanal followed a similar pattern of formation in both emulsions. Even though 2-pentylfuran formation was more evident in CTT emulsions, generation of wider range of linoleic acid products detected in MWT emulsions indicate that this fatty acid was utilized as a substrate both by LOX activity and radical reactions resulting in higher diversity of linoleate volatiles. Accordingly, in MWT emulsions this broader range of volatiles was a reflection of ample hydroperoxide formation in comparison with CTT emulsions. In common with all emulsion groups, hexanal, as the major linoleate oxidation product, was the most prevailing volatile. However, apart from hexanal, the dominance of detected volatiles showed variance between different emulsion groups. The most evident discrepancy between UT emulsions and other emulsions was the emphasized formation of ketones in MWT, CTT, and SP emulsions. This can be attributed to the mechanism through which ketones arise. Ketone formation is usually observed via radical recombination reactions of alkyl, peroxyl, and alkoxy radicals while LOX-catalyzed oxidation initially does not rely on free radical-dependent reactions (Schaich et al. 2013). Thus detection of ketones was less emphasized in UT emulsions. In SP emulsions volatile formation was more pronounced during the later days of storage at 37 °C. This was observed in steep increase of detected volatile compounds. Combined with the continuous propagation of CD formation, these results showed that generation of stable end products of oxidation was delayed in SP emulsions compared to other emulsions. Furthermore, due to absence of the LOX pathway, lipid oxidation was expected to proceed dependent on autoxidation mechanism in which radical-initiated oxidation prevailed. This result was

also supported by the higher abundance of ketones observed in SP emulsions in comparison to MWT and CTT emulsions.

6.2.3 PROTEIN OXIDATION AND THE INFLUENCE OF LIPID CO-OXIDATION

Oxidation of proteins resulted in significant degradation of Trp fluorescence in emulsions stabilized by quinoa and amaranth proteins (II). During the storage period peak maxima of fluorescence spectra shifted from 343 to 353 nm in quinoa protein-emulsions and 344 to 358 nm in amaranth protein-stabilized emulsions. This phenomenon of red shift in the spectra is associated to an increase in the polarity of the environment (Möller and Denicola 2002). It is known that hydroperoxides formed during lipid oxidation are more polar in nature compared to the lipid source such as triacylglycerides and therefore migrate to the interfacial layer where proteins are located (Schaich et al. 2013). Moreover, oxidative modification of proteins leads to increased exposure of tryptophanyl residues to the polar aqueous environment (Kim et al. 2002).

In emulsions with quinoa proteins the highest loss of fluorescence took place between day 0 and 1 while fluorescence intensity remained level during the last days of storage. This higher initial rate of protein degradation point to a radical-driven pathway of reactions that are dominant at the onset of oxidation. This observation of rapid protein oxidation may be the result of generation of reactive species between proteins and lipids which cause the oxidative damage of proteins in parallel to lipids. Berton et al. (2012b) reported that protein modifications may precede lipid oxidation and afterwards proceed via free radical transfers between proteins and lipids, propagating the co-oxidation. In this case it is possible that protein radicals were formed prior to lipid species and reinforced the consequent formation of lipid oxidation products leading to lower physical stability of emulsions. The unchanging levels of Trp fluorescence towards the end of the storage point to almost complete oxidation of Trp residues while it does not eliminate the involvement of radicals of quinoa proteins in ongoing oxidation. This could be observed in increasing amounts of CD in protein-stabilized emulsions during the last days of oxidation while LC-MS formation in Tween®20-emulsions slowed down. Emulsions stabilized with amaranth proteins exhibited a different route of protein oxidation where the highest extent of protein damage occurred between days 1 and 4. This shows that while quinoa proteins were rapidly involved in oxidative reactions, degradation of amaranth proteins was relatively delayed. The discrepancy between the rates of protein oxidation was also paralleled in formation of secondary lipid oxidation products which was more pronounced in quinoa protein-stabilized emulsions. One of the reasons for the difference may have

stemmed from the structural differences between quinoa and amaranth proteins. Drzewiecki et al. (2003) elucidated that amaranth proteins contain a higher fraction of α-helices compared to quinoa proteins in which β-sheets cover a higher percentage. Fujiwara et al. (2012) explained that the propensity for Trp residues are higher towards β-sheet conformation than α-helices. Thus the location and availability of Trp residues in secondary structure of quinoa proteins stabilizing lipid droplets may have provided a higher exposure for Trp degradation. Another reason for the difference may be explained by the higher SSA values observed in freshly prepared quinoa protein-emulsions where a higher interfacial area translates as more available area for oxidative reactions.

In both quinoa and amaranth protein-stabilized emulsions, protein oxidation and lipid oxidation proceeded in parallel, thus supporting the significant influence of protein and lipid co-oxidation and its effects on physical stability. It was previously reported that although moderate oxidation of proteins may increase the emulsifying activity, extensive oxidation results in deterioration of interfacial protein network and cause aggregation (Kong et al. 2013; Liu et al. 2015; Berton-Carabin et al. 2016). Protein oxidation correlated also to physical stability as observed via measurements of droplet size distribution of emulsions stored at 6 °C. The protein-stabilized emulsions were found to exhibit a better stability at lower temperature at which the presence of smaller droplets produced in freshly prepared emulsions was still persistent on day 7. At lower temperature the extent of oxidative modifications would be anticipated to be lower due to delayed initiation of radical-driven autoxidation. Furthermore, as was the case at 30 °C, emulsions with quinoa proteins had lower stability compared to amaranth protein-emulsions. In addition to other results presented, this indicates that emulsions stabilized with amaranth proteins exhibit higher stability in terms of protein and lipid oxidation as well as physical stability.

On the other hand, measurements of Trp fluorescence in separated phases of the emulsions prepared with faba bean proteins provided information on the extent and course of protein oxidation in aqueous phase and the interfacial layer. The discrepancy between the peak maxima wavelengths of Trp fluorescence in adsorbed and unadsorbed proteins was reported previously (Salminen et al. 2010; Berton et al. 2012b) and was due to the conformational differences of the proteins stabilized in continuous phase and cream phase as well as the exposure of Trp residues in these conformations (Munishkina and Fink 2007).

Oxidative degradation of interfacial proteins in MWT emulsions was more emphasized compared to CTT emulsions. This could be due to the generation of lipid peroxyl and alkoxyl radicals from hydroperoxide decomposition that was observed after day 1 in MWT emulsions. These highly reactive species are known to be involved in radical transfer reactions

between proteins and lipids (Schaich 1980). Apart from lipid radical-initiated oxidation, protein radicals already present may also have triggered oxidation at the interface. Fan et al. (2016) previously reported that the excitation of water molecules during microwave treatment developed protein radical formation in a larger scale than during oven heating. Therefore, free protein radicals may have caused oxidative damage to interfacial proteins in MWT emulsions as well as promoted lipid oxidation via free radical chain reactions. In adsorbed proteins of CTT emulsions an increase in Trp fluorescence intensity was recorded between day 0 and day 1. Although the reason behind this rise was not exactly clarified within the model study, this observation may be due to the conformational changes exerted on the proteins during the thermal treatment. Proteins in CTT emulsions may have been in the process of reaching an equilibrium between folded and unfolded state (Eftink 1994) while stabilizing the lipid droplets during the first day of storage and causing a delayed fluorescence of Trp residues. Following day 1, degradation of interfacial proteins of CTT was observed in a steady rate throughout the rest of the oxidation period indicating the involvement of lipid radical species as autoxidation progressed. Meanwhile, adsorbed proteins in UT emulsions displayed the least amount of oxidative degradation although early detection of abundant lipid oxidation products was evident in these emulsions. This is attributed to the relative absence of radical-driven reactions at the beginning of the oxidation. Accordingly, loss of Trp fluorescence was delayed as radical generation occurred when hydroperoxides decomposed. Oxidation of adsorbed proteins in SP emulsions took place in a faster and greater extent than the other emulsions. This observation was in accordance with the high volatile formation in later stages of the storage. Oxidation in SP emulsions was expected to follow autoxidation pathway due to lack of LOX activity. Since proteins were susceptible to radical attacks from reactive lipid species the competition for the radical sources may have promoted a prolonged propagation stage which caused a delay in formation of secondary lipid oxidation products. This effect at the beginning stages of oxidation would be absent in faba bean protein-emulsions due to the presence of residual enzymatic activity.

Meanwhile, unadsorbed proteins were found to be oxidized in a lesser extent compared to the interfacial proteins in MWT, CTT, and SP emulsions. This was in accordance with the results of previous studies (Rampon et al. 2001; Berton et al. 2012b) which confirmed that interfacial proteins are more prone to oxidative modification than continuous phase proteins. Among these emulsions, aqueous phase proteins in MWT emulsions were oxidized in the highest extent. As mentioned before, pre-existing protein radicals due to microwave treatment may be the reason for this observation in aqueous phase proteins while lack of reactive lipid radicals prevented further oxidation in comparison with interfacial proteins. Oxidation of unadsorbed

proteins in UT emulsions followed a similar trend of moderate decrease as other emulsions until day 4, which was then followed by a remarkable protein degradation as observed in extensive loss of Trp fluorescence intensity. This observation can be explained by the generation of lipid oxidation products in overwhelming abundance in later stages of storage. Oxidation of lipids yield compounds such as hydroperoxides and secondary oxidation products that are more polar in nature compared to triacylglycerides and fatty acids (Schaich et al. 2013) and migrate to the polar solvent phase thus becoming involved in oxidative interactions with unadsorbed proteins.

In study III emulsions prepared with proteins from conventional thermal-treated faba beans provided better oxidative stability in lipid and protein components, whereas overwhelming influence of LOX activity caused extensive oxidation in emulsions stabilized by proteins from untreated faba beans. Interfacial proteins were modified in a higher scale than the unadsorbed proteins due to their oxidative interactions with reactive lipid species while advanced levels of lipid oxidation observed in UT emulsions caused oxidative degradation of continuous phase proteins in later stages of storage. Interactions between proteins and lipids were found to be influential on the overall oxidative stability of emulsions and in particular on the location of proteins present in these systems. Therefore optimization of protein compositions as well as pre-treatment conditions utilized in emulsion-making may yield in products with better oxidative stability.

In these model studies, oxidative degradation of proteins was more stressed in emulsions prepared with quinoa and amaranth proteins (II) than faba bean protein-stabilized emulsions (III), even though the experiment was carried out at a lower temperature with lower lipid content. The formation of hydroperoxides was also found to have proceeded in a higher extent in emulsions of study II than those monitored in study III. Thus, during the propagation stage where radical transfer reactions take place between proteins and lipids, oxidation advanced greater in quinoa- and amaranth-stabilized emulsions than faba bean protein-stabilized emulsions. This result demonstrates the significance of the degree of protein oxidation on the overall oxidative course of the model system and the consequent outcomes.

7 CONCLUSIONS

Involvement of proteins within oxidation reactions in food systems significantly influences the course of lipid oxidation. The overall progression of oxidative modifications relies on characteristics and composition of proteins and lipids. This thesis study focused on determining these modifications and investigating the development of oxidation in model studies.

Utilization of LC-MS/MS technique coupled with UV and fluorescence detectors proved to be useful and accurate in characterization of adducts formed between tryptic β-Lg peptides and lipid oxidation product MDA. Two distinct Schiff base adducts were observed located at the amino groups of N-terminal amino acids as well as His and Gln side-chains. One of these compounds was the 3-amino-2-propenal derivative (enaminal) observed with a mass increment of the unmodified peptide with a value of +54 Da, while the other was the fluorescent dihyrdopyridine-type (DHP) adduct with a mass increment of +134 Da. MDA was highly reactive towards these nucleophilic amino groups and the modifications could be detected as adduct formations early in the incubation period. The higher storage temperature increased the abundance of adduct formation, however caused the breakdown of enaminal compound early in the incubation period while the fluorescent DHP-type adduct showed stability at both temperatures.

Emulsions stabilized with quinoa and amaranth proteins showed lower physical stability than Tween®20-stabilized emulsions. This result was also reflected in lower oxidative stability of lipids in the emulsions with quinoa and amaranth proteins. While hydroperoxide formation followed a similar trend in all emulsions, rate of conjugated diene generation increased in protein-stabilized emulsions after day 4. Release of volatiles detected via SPME-GC-MS also showed that oxidation advanced further in protein-stabilized emulsions. On the other hand volatile aldehydes 2-heptenal, 2-pentenal, and 2,4-heptadienal formed in more abundant levels in Tween®20-emulsions. This observation was attributed to the susceptibility of these volatiles to further oxidative reactions in advanced stages of storage. One example of volatiles arising from oxidation of lipid aldehydes, 5-pentyl-2(5H)-furanone was detected in abundance in protein-stabilized emulsions presence of which remained in minor levels in Tween®20-emulsions. Quinoa proteins underwent rapid oxidative modifications observed in the loss of Trp fluorescence within one day of storage while oxidation of amaranth proteins was relatively delayed. Higher oxidative degradation of quinoa proteins was also reflected in the physical stability of emulsions analyzed both at 30 °C and 6 °C. While the expected lower levels of oxidation at 6 °C caused a

remarkable increase in physical stability of emulsions with proteins, higher stability of emulsions with amaranth proteins over quinoa protein-containing emulsions was established. In conclusion, the extent of protein oxidation was one of the important factors that determined the physical stability of emulsions in addition to the progress of lipid oxidation.

Microwave (MWT) and conventional thermal treatments (CTT) of faba beans remarkably decreased the scale of oxidation in emulsions stabilized with proteins extracted from pre-treated and untreated faba beans. The effectiveness of the pre-treatments against the lipoxygenase (LOX) enzyme activity was higher in CTT compared to MWT. LOX presence was influential on the course of lipid oxidation where the propagation of radical-driven reactions was less pronounced due to enzymatic activity. This point was supported by the analysis of lipid oxidation products which showed that ketone formation was more prevalent in MWT, CTT, and SP emulsions. Ketones were reported to particularly arise as a secondary oxidation products of lipid species during radical recombination reactions. CTT emulsions displayed higher oxidative stability than MWT emulsions due to the remaining LOX activity in the latter. Furthermore, emulsions were separated into their continuous and cream phases to study the oxidative modifications of adsorbed and unadsorbed proteins. Proteins utilized in stabilizing lipid droplets at the interface were oxidized in a larger scale than those found in continuous phase of MWT, CTT, and SP emulsions due to the majority of protein-lipid co-oxidation reactions taking place at the interface. At later stages of oxidation, unadsorbed proteins in UT emulsions underwent extensive degradation most likely triggered by interactions with the more polar lipid oxidation products that migrated towards aqueous phase. This study showed that the development of oxidation in emulsions was dominated by the selectivity in the oxidation pathway influenced by the type of proteins as well as the presence and lack of lipoxygenase.

These model studies demonstrated that the influence of proteins and involvement of protein oxidation in overall oxidative stability of systems that contain proteins and lipids as major components are highly significant. Focus on future studies may include optimization of solutions that aim to inhibit oxidative degradation of both proteins and lipids. Further investigations of the oxidative stability may be conducted in more complex systems that involve other constituents such as carbohydrates, antioxidants and surfactant emulsifiers.

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